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The development of a new 15N-stable isotope probing method for the quantification of biological nitrogen fixation in soils

School of Chemistry

The development of a new ^{15}N-stable isotope probing method for the quantification of biological nitrogen fixation in soils

by

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Abstract

Biological nitrogen fixation (BNF) performed by diazotrophs is vital to our understanding of ecosystem functions, as nitrogen (N) is commonly a limiting factor in primary productivity. Yet, significant limitations remain in our knowledge of the controls and rates of this process. To address this problem, we developed a novel ^{15}N-stable isotope probing method via gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) for the quantification of BNF in soils. Through initial method development on both symbiotic and free-living diazotrophs in clover root nodules and peat, respectively, the key properties governing N_2 fixation were identified and more comprehensively understood. Refinements were then made to the protocol and retested on peat: longer incubations and the addition of an energy source proved successful as the level of ^{15}N enrichment, i.e. N_2 fixed, was greatly enhanced. Further method refinements were carried out on British grassland soils, where root exudates were found to be the most effective source of energy in fuelling N_2 fixation. The process is also limited by the availability of N within the environment – whether through inputs of N fertiliser or atmospheric N deposition. Lastly, the validated method was applied to a more diverse environment by assaying a range of soils from southwest China, in order to assess the responses of free-living diazotrophs in a recovering ecosystem. Results correlated well with vegetation recovery phases where N_2 fixation increased in order of: sloping farmland > recently abandoned farmlands > secondary forest. Anomalies could be explained through other conditions such as soil moisture levels and inactive *nifH* genes. Taken together, the successful development of a compound-specific GC-C-IRMS method to quantify BNF allows an improved interpretation of the process in a highly sensitive manner – thereby enabling further implications for sustainable agriculture and food security.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: ..... DATE: .....

Acknowledgements

"If I have seen further, it is by standing upon the shoulders of giants." – Sir Isaac Newton

Newton's cherished metaphor may be a cliché, but it still perfectly embodies what I hope to convey. But rather than limiting the definition to a scientific context, I would like to open it to everyone who has kindly enabled my journey in any way. As with everything else I've done, this thesis would not have been possible without the sincere support of many people:

I am greatly indebted to my supervisor, Professor Richard Evershed, for his advice and support since day one. Thank you for inspiring me with your unfailing enthusiasm in research and for always asking the real questions. Most importantly, thank you for giving me a chance. The past two years has been an invaluable learning experience and none of this would have ever happened without your help and guidance.

This research was funded in part by the SPECTRA partnership. I am grateful to Heather Buss for taking the time to track down the Chinese soils for me and allowing me to use her lab and its facilities. A special thanks goes to Adam McAleer for all his kind support and sharing with me his expertise on both gas systems and peatlands – much of the method development could not have been accomplished without him. Also, thank you to Rob Griffiths for directing us to the magnificent Exmoor peat bogs that set-off this journey.

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I would not be anywhere without the unconditional love and support of my mum and family. My mum has always been my rock. In her selflessness, she has allowed me the freedom to be who I am and do whatever I aspire to do. She is the kinder, stronger and much more confident person that I strive to be. I am also very grateful to my grandparents for their unceasing comfort and warm presence – thank you for always opening your arms for me. And to Jiaming, thank you for being my home away from home. To all my friends, thank you for also being a part of my family. It takes a village to raise a child – and apparently, a thesis as well.

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1 Introduction

1.1 The nitrogen paradox

In the 18th Century, the French chemist Antoine Laurent Lavoisier initially gave nitrogen (N) the name *azote*, denoting "no life." Unlike oxygen, the other key constituent of air, this gas was seemingly unable to support the metabolism of living organisms autonomously. However, major strides in our knowledge of N since then has tinged the original name with irony. N is now widely acknowledged as one of the principal nutrients vital for the survival of plants and all other forms of life – being a critical component of various biomolecules, including DNA, proteins, and chlorophyll (Brill, 1977; Hirsch and Mauchline, 2015).

Although 78% of the Earth's atmosphere is made of nitrogen (N_2), another paradox lies in how the element simultaneously exists in abundance and, yet, is a scarce nutritional resource (Vitousek *et al.*, 1997; Hirsch and Mauchline, 2015). This mismatch is due to the chemistry of the molecule: nitrogen exists in air as a diatomic gas (N_2), where the two atoms are joined by an especially strong triple bond. As a substantial amount of energy is required to break this bond, N in this form is so inert that it is inaccessible to the majority of organisms (Brill, 1977). Thus, despite having an atmospheric reservoir of 3.7×10^9 Tg (Fig. 1.1), N is only sparsely available to much of the biological world and frequently limits primary productivity and the functioning of various ecosystems (Sorai *et al.*, 2007).

In order to enter biological systems and be assimilated by primary producers, N must first be 'fixed'. That is, it must be drawn from the air and bonded with certain elements, namely hydrogen or oxygen, to form inorganic N compounds required for cellular growth and metabolism (Brill, 1977). This interchange between inert N_2 gas within the atmosphere and 'reactive' N usable by plants and other organisms is largely directed by microbial activities and performed by specialized prokaryotes (Franche *et al.*, 2009; Hirsch and Mauchline, 2015). Such transformations of N and the cycling of N in soil are collectively referred to as the biological N cycle.

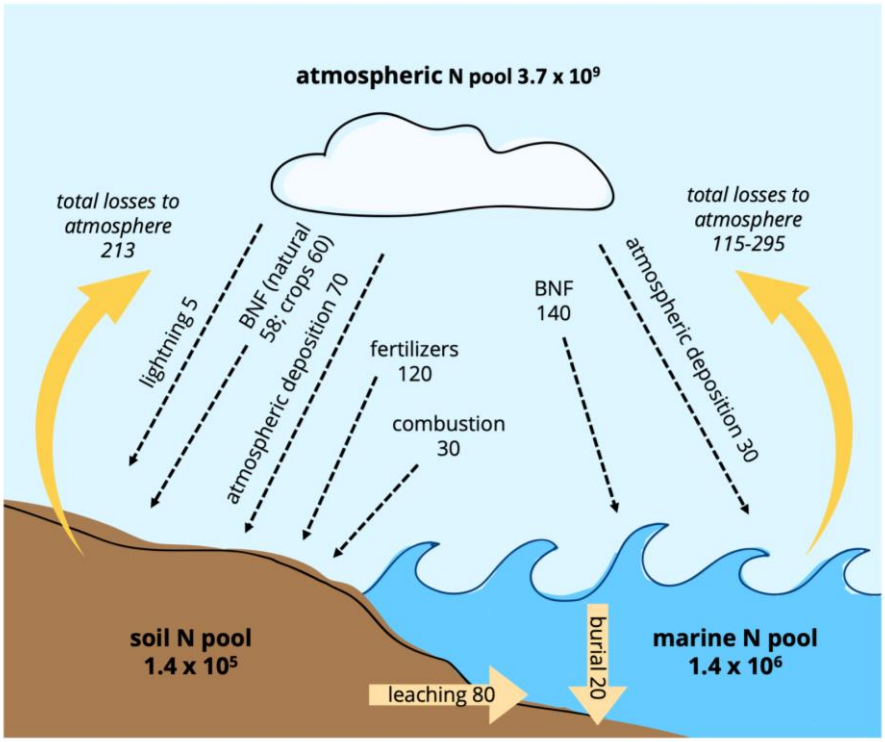


Figure 1.1. The processing and exchanges of nitrogen in the atmosphere, terrestrial, and marine systems – including major N reservoirs in Tg, and fluxes in Tg yr^{-1}. (Flux data from Fowler *et al.*, 2013. N reservoir data from Sorai *et al.*, 2007; Ward, 2012).

1.2 The biological nitrogen cycle

The simplified biological N cycle can be regarded to begin and end with N_2 (Fig. 1.2). Microorganisms control the N cycle, opening with N_2 fixation, where N_2 is converted to ammonia (NH_3). Nitrification, the next major step, oversees the oxidation of NH_3 to nitrite (NO_2^-), and then nitrate (NO_3^-). Subsequent denitrification reduces it back to N_2, largely leading to a net loss of N to the atmosphere (Postgate *et al.*, 1980). Thus, the cycle is completed as bioavailable N is removed and returned back to the atmosphere (Bernhard, 2010; Hirsch and Mauchline, 2015). These oxidation-reduction reactions, which convert N between its stable states allow organisms to obtain and produce energy, and as a result, also regulate the natural distribution of N on Earth (Ward, 2012).

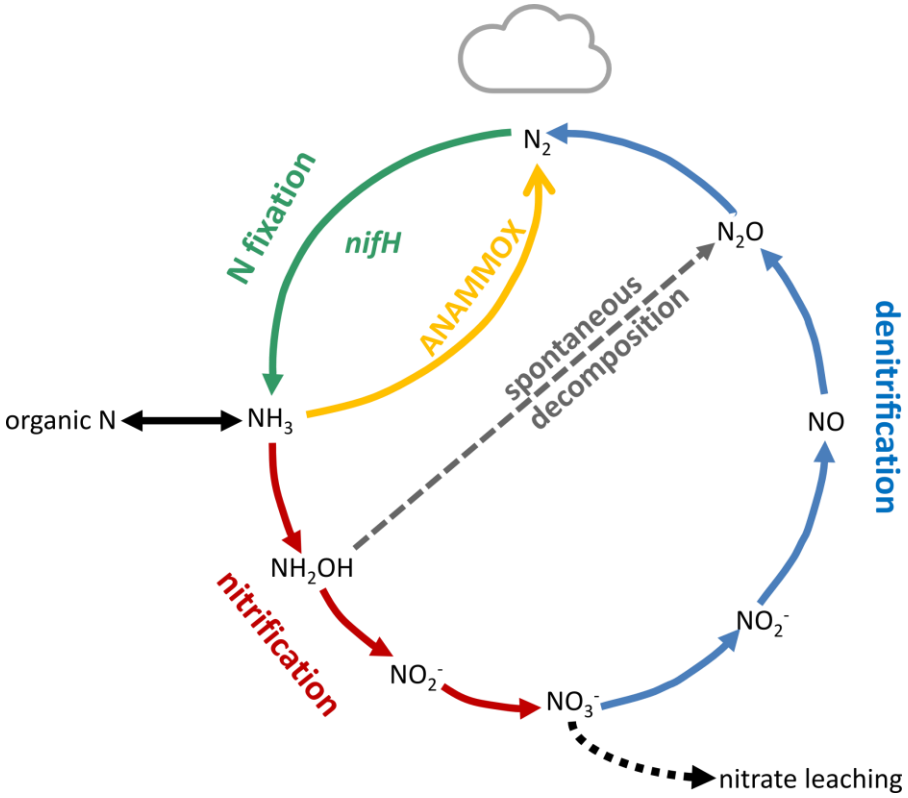


Figure 1.2. The biological N cycle. Following the chemical conversion of N through the main processes of nitrogen fixation, nitrification, and finally, denitrification. (Adapted from Hirsch and Mauchline, 2015).

1.3 Nitrogen fixation

The onset of the biological N cycle, N_2 fixation is the process of converting N_2 into NH_3 – allowing N to be further transformed into a broad range of N-containing biochemicals: amino acids, nucleic acids, and other compounds of biological importance. In most environments, however, the availability of fixed N is often what limits plant productivity (Dixon and Kahn, 2004; Franche *et al.*, 2009). Consequently, not only is N_2 fixation of immense importance to the environment and global agriculture, it also plays a vital role in the N cycle by replenishing the biosphere's total N content and offsetting losses incurred from denitrification (Dixon and Kahn, 2004).

As N_2 gas is an extremely stable compound, a substantial amount of energy is required for N to be utilised by organisms. The whole process of N_2 fixation requires 8 electrons, and the energy expenditure is estimated to be at least 16 ATP for every molecule of N_2 reduced (Eq. 1.1; Fisher and Newton, 2002). For this reason, only an exclusive group of prokaryotes have the ability to perform this energy intensive reaction (Bernhard, 2010; Hirsch and Mauchline, 2015). The

mechanism itself is strictly regulated on numerous levels and much of N_2 fixation relies on the availability of exogenous carbon sources for ATP (Stewart, 1969).

$$N_2 + 16\,ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Equation 1.1. The overall stoichiometry of N_2 fixation, under optimal conditions. ATP stands for adenosine triphosphate – the molecular unit of energy in a cell. Once it has been expended in metabolic processes, ATP can be converted into adenosine diphosphate, or ADP. Inorganic phosphate is represented by P_i.

1.3.1 Sources of fixed N

The compensating conversion of gaseous N_2 to forms which are accessible to plants and animals occurs through three main routes. One not particularly effective way is via lightning (Fisher and Newton, 2002). On the other hand, industrial N_2 fixation producing man-made fertilisers has grown exponentially since the discovery of the Haber-Bosch process – representing a major dichotomy between the needs of the human population and the planet's natural wellbeing (Fowler *et al.*, 2013). Another anthropogenic activity also involves N_2 fixation induced through the combustion of fossil fuels and car engines, prompting increased inputs of N oxides, NO_x, in the atmosphere. The third, and final, route of widespread interest is biological nitrogen fixation (BNF). N introduced by this route distinguishes itself by taking place at the microbial level and is by far the most significant source of fixed N derived from the natural world (Bernhard, 2010).

1.3.1.1 Lightning

N_2 fixation via electrical discharges, or lightning, is wholly an abiogenic process. A form of atmospheric deposition, the enormous energy generated by lightning can split the triple bond in N_2 and therefore enable the creation of additional chemical species. This also includes the formation of NO_x, allowing reactive N to be introduced and deposited down through the troposphere and onto the land. In rain, these NO_x compounds dissolve to form NO_2^- and NO_3^-, which are transported to earth. Nevertheless, Fowler *et al.* (2013) calculates a value of only 5 Tg N produced from lightning annually (Fig. 1.1). Despite the high number of lightning flashes globally, it is not a significant route of N_2 fixation for primary producers (Fisher and Newton, 2002).

1.3.1.2 Anthropogenic N_2 fixation

In the recent past, industrial N_2 fixation through the production of chemical fertilisers have undisputedly changed the scope of food production – becoming an indispensable element of current agricultural practices (Bohlool *et al.*, 1992). Once natural ecosystems are perturbed and brought under cultivation, the areas typically become N-limited, and N-fertiliser is often introduced (Postgate *et al.*, 1980). The N administered in modern agriculture stems from atmospheric sources; but in contrast to the natural process of BNF, agricultural N is fixed on an industrial scale using the Haber-Bosch process. Originally pioneered by Fritz Haber and later Carl Bosch during the First World War, this synthetic method of N_2 fixation involves the combination of atmospheric N_2 with hydrogen gas (H_2), typically obtained from petroleum or natural gas sources. With the help of a catalyst, the two components are combined under high pressure and temperature, resulting in the reduction of N_2 to NH_3 (Eq. 1.2; Fisher and Newton, 2002). Fertilisers are then made following an acid-base reaction; for example, the reaction of NH_3 with nitric acid (HNO_3) to produce the fertiliser, ammonium nitrate NH_4NO_3.

$$N_2 + 3H_2 \xrightarrow[\text{Fe catalyst}]{\text{450°C ; 200 atm}} 2NH_3$$

Equation 1.2. The chemical reaction of the Haber-Bosch process, and its conditions.

Nonetheless, it has attracted much attention that the increasing production and usage of N-fertilisers also fuels financial problems. A Haber plant incurs significant capital cost and technical upkeep to setup and run – requiring an industrialised and highly developed nation for its execution. In reality, however, the main demand for N-fertilisers lies in developing countries; thus, adding further transportation costs to the market price (Postgate *et al.*, 1980). The production of industrial fertilisers expends non-renewable fossil fuel resources as a feedstock for natural H_2 gas and a source of energy. Limitations in the availability of such reserves will inflate the true cost of N-fertiliser and subsequently, food.

N-fertilisers are also viewed as a pollutant. To achieve the highest yield, farmers often supply more than double the fixed N needed by a crop. The applied N is easily converted to mobile NO_3^-, which leaches into ground and surface waters, leading to environmental issues ranging from the contamination of freshwater supplies to eutrophication (Bøckman, 1997; Fisher and

Newton, 2002). Globally, 50–70 % of the total amount of N-fertiliser entering crop systems was reported to be lost to the immediate environment (Cassman *et al.*, 2002; Schlesinger, 2009; Ladha *et al.*, 2016). Furthermore, atmospheric N_2 is also inadvertently fixed through other anthropogenic activities – especially in the combustion of fossil fuels, the production of electricity and industrial undertakings (Postgate *et al.*, 1980). These have contributed to increased N deposition, and therefore prompted situations where N is in excess. When accumulated N deposition surpasses the nutritional requirements of the ecosystem, plant productivity and the environment also become adversely affected.

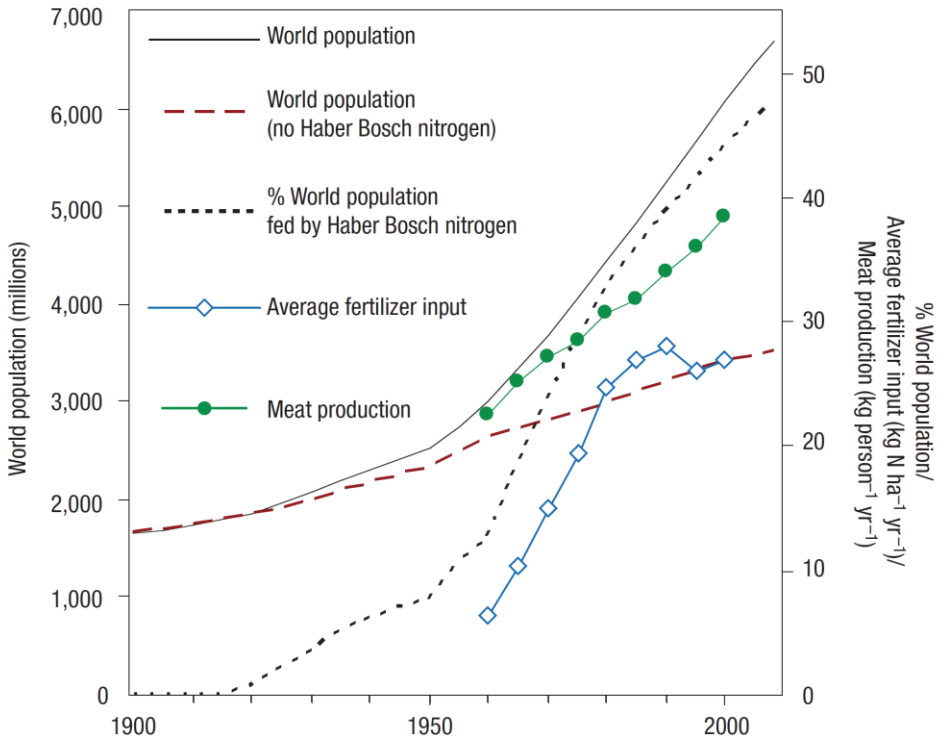


Figure 1.3. Global human population and N use trends throughout the 20th Century. The total world population is shown as the solid dark line, while the long-dashed red line an estimate of the total number of people living in a world without industrially fixed N. The short-dashed line is the % of the global population that is fed by N from the Haber-Bosch process. Also shown in blue and green are the average N fertiliser input per hectare of farmland and the increase in meat production per capita, respectively. (Taken from Erisman *et al.*, 2008).

The full extent of anthropogenic in relation to natural sources of fixed N has increased the global cycling of N by almost two-fold over the last 100-years (Fowler *et al.*, 2013). Human N creation is currently estimated to be roughly 210 Tg N yr^{-1} from the combination of various anthropogenic activities (Galloway *et al.*, 2013). At the start of the 21st Century, almost 50 % of the entire world

population are likely to have had their diet dependant on fertiliser N (Fig. 1.3). The Haber-Bosch process alone was reported to produce 120 Tg N in the form of NH_3 per year, in 2010 (Fowler *et al.*, 2013). Needless to say, while human modification of the biological N cycle comes with important benefits in feeding billions of people, changes in the supply of a major nutrient such as N also has detrimental impacts on climates and the functioning of ecosystems (Erisman *et al.*, 2008). For this reason, a practical equilibrium between the two ends must be sought.

1.3.1.3 Biological N_2 fixation by diazotrophs

Discovered in the late 19th Century, BNF has since been of increasing ecological interest as the main non-anthropogenic input of fixed N to be used by living organisms (Franche *et al.*, 2009). The microbial conversion of N_2 into NH_3 is only driven by prokaryotic microorganisms known as diazotrophs. This group of specialised organisms are capable of fixing atmospheric N_2 and do not require any other sources of N to survive (Dixon and Kahn, 2004). Diazotrophs can be further separated into sub-categories depending on their function as symbiotic, associative, or free-living species. Crop rotation schemes involving beans and clovers are a common example of diazotrophs at work. The method has been practiced by farmers over many centuries as a method of 'recovering' soil that had been nutrients-depleted due to continuous cultivation. It was not until later that studies localised the activity to a N-fixing symbiosis by bacteria-filled nodules located on the roots of leguminous crops (Pfau *et al.*, 2018).

BNF may take place in both terrestrial and marine ecosystems, where global BNF values are estimated to be around 58 and 140 Tg N yr^{-1} (±50 %), respectively (Fig. 1.1; Vitousek et al., 2013). The large range of uncertainties reflect on the various complications arising in the deduction of these component terms. For example, hydrological losses of N from land systems and spatial variability within ocean systems may have led to some cases of overestimation (Fowler *et al.*, 2013).

The advantages of BNF have been demonstrated in various areas, ranging from agronomic to environmental. An overriding strength of BNF is the direct supply of atmospheric N_2 to the soil, and the ensuing transfer of this N to relevant primary producers. Accordingly, the significant benefits of rotating legumes into cropping systems arise from the amount of N relayed below-ground – largely through the decomposition of their nodules and roots (Ledgard and Steele, 1992). Diazotrophs exist naturally in nature and offer a more economic form of N for crops,

rather than sole reliance on industrial fertilisers. The substitution of chemical-based technologies with a biological method also delivers environmental benefits in decreased levels of NO_3^- pollution in ground-waters and reduced production of greenhouse gases from fossil fuels (O'Hara, 1998).

There are still many factors affecting the extensive use of BNF today. Present day activity in the research of N fixation is fuelled by the knowledge that food production is directed by the input of N. The current challenge for scientists and farmers is to maximize the effective and efficient practice of BNF in agricultural systems. This involves both increasing N input from biological fixers and minimising losses of N (O'Hara, 1998). Accurate quantifications of BNF rates should also be established as these are vital in and testing relevant controls and hypotheses. Overall, the components of BNF needs to be picked apart and thoroughly analysed and understood – in order to be reassembled and used in a wider context.

1.4 Diazotrophs

Microbes play a leading role in the biological N cycle and only particular bacteria and archaea – referred to as diazotrophs – are able to biologically reduce the N_2 triple bond to NH_3 for assimilation by plants. Within these diazotrophs, the biological relationships formed by N-fixing organisms primarily extend from obligatory symbiotic to free-living, as well as an assortment of associative symbioses in between (Hardy and Havelka, 1975).

1.4.1 Symbiotic diazotrophs

Symbiotic N-fixers need to form a close association with a host to fulfil BNF. Thus, most associations in this category follow very precise and complex mechanisms to help sustain the symbiosis (Bernhard, 2010). Symbiotic BNF commonly occurs through root-nodule relationships; the best-understood relationship of this type occurs between leguminous plants and a form of root-nodule bacteria known as rhizobia. These N-fixing bacteria reside in the outgrowths – or nodules – of the plant's roots, allowing a maximised transfer of fixed N from the rhizobia to the participating host plant (Fisher and Newton, 2002).

For symbiotic BNF to work, a relationship between the host and symbiont bacteria has to first be established through nodule development (Fig. 1.4). This involves an intricate series of processes, beginning with the survival and successful colonisation of active strains of rhizobia in the soil. As the bacteria generally subsists in soil as free-living heterotrophs, this first requirement is largely dependent on adequate soil and environmental conditions. In cases where root nodules fail to develop due to a low populace of effective and compatible rhizobia in the soil, the addition of appropriate strains may be introduced via inoculation (Ledgard and Steele, 1992).

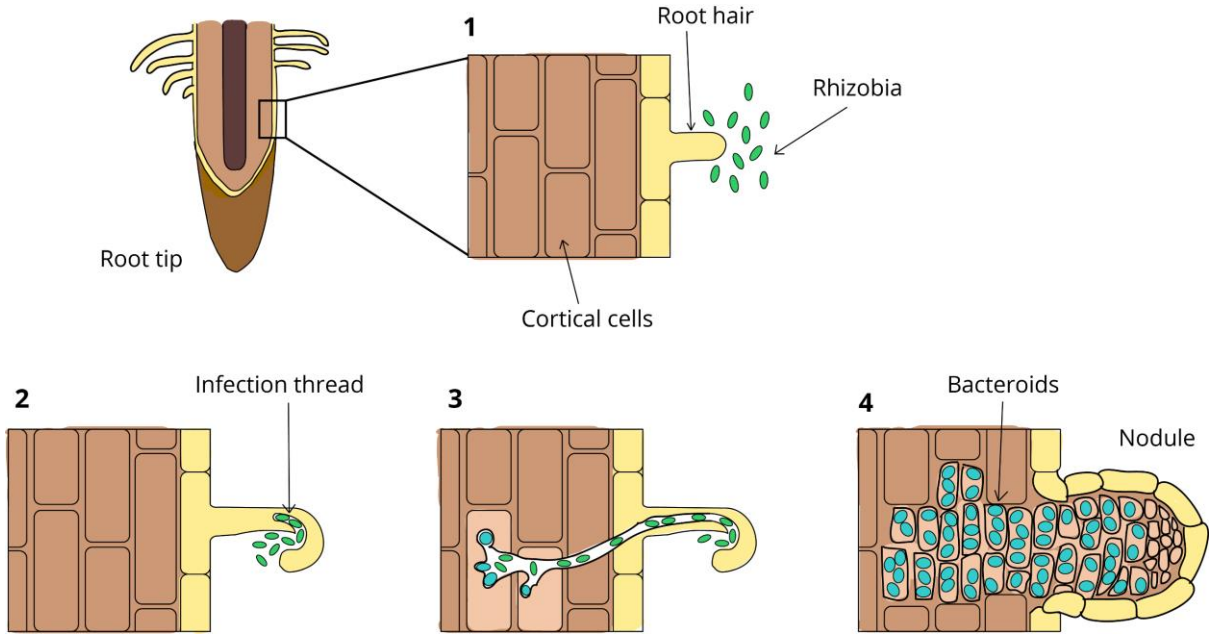


Figure 1.4. Root nodule development. In the first step, the root hairs of a legume plant release flavonoids which induce nearby rhizobia. The N-fixing bacteria secretes Nod factors in response, instigating a series of signals which cause the root hair to curl. An infection thread is subsequently formed, and the rhizobia may now enter into the plant's cortical cells. Finally, root cells begin to divide and bacteroid formation from the invading bacteria completes the nodulation process.

As with all symbiotic relationships, the mutualism between root nodule bacteria and their particular host legume is an unceasing process, reliant upon a thoroughly regulated chemical dialogue ensuing from both sides (O'Hara, 1998). Compatible host plants benefit from this molecular association as the rhizobia exports fixed N that is required by its host. The plant reciprocates by providing a sheltered environment and supplying the bacteria with C sources to fuel the energy-intensive reaction (Mylona *et al.*, 1995; Cleveland *et al.*, 1999).

However, while there may be a plethora of root nodules appearing on the host plant, only a limited number actually serve as effective N-fixing strains (Pfau *et al.*, 2018). Rhizobia which take plant resources but fix little-to-no N_2 after the formation of root nodules commonly exist; if plants were to treat all nodules similarly without penalisations, those that invest very little to N_2 fixation will be favoured by natural selection (Kiers *et al.*, 2003). Thus, it has been indicated that hosts can monitor their symbionts' performance and impose sanctions on ineffective nodules in order to secure mutualism. This may be through restriction of the supply of energy-rich compounds, such that plant resources are only routed to 'working' nodules (Mus *et al.*, 2016).

1.4.2 Free-living diazotrophs

As denoted by their title, non-symbiotic, or free-living N-fixers are able to fix N_2 without reliance on other organisms (Hardy and Havelka, 1975). A majority of diazotrophs fall into this category; they do not form a relationship with plants or other animals, and live freely within the soil (Fisher and Newton, 2002). Over short periods of time, however, free-living diazotrophs rarely fix large amounts of N_2 since they must find their own sources of energy to carry out the reaction. While energy is supplied to symbiotic N-fixers by the host plant, exogenous C required by free-living diazotrophs is often found naturally in limitation. Nevertheless, conditions can be modified, and exceptions can occur. For example, as free-living N-fixers are able to use a wide range of energy-rich sources, the rhizosphere and soil surface layers often provide environments of high C and low O_2 tension needed for them to function most efficiently and fix significant quantities of N that may then be exported to their surroundings (Stewart, 1969).

Free-living N-fixing bacteria are phylogenetically more diverse and includes both heterotrophs and autotrophs. Some of the best-known N-fixing heterotrophs include *Azospirillum*, that is found in the rhizosphere of major cereal crops, and *Azotobacter*, which can fix N aerobically. Free-living N-fixing autotrophs include cyanobacteria. These bacteria are not only ubiquitous in tropical soils, but also occupy flooded rice fields – contributing from 5–25 kg N ha^{-1} yr^{-1} (Kahindi *et al.*, 1997). Under specific conditions, free-living diazotrophs may fix a combined total of up to 60 kg N ha^{-1} per year (Bürgmann *et al.*, 2004). Although symbiotic diazotrophs contribute to a higher rate of N_2 fixation, the global importance of free-living species lies in their large numbers and broad distribution. They may possibly provide the bulk of fixed N in environments spanning from tropical forests to temperate grasslands to barren deserts (Angel *et al.*, 2018). Their overall contribution to N_2 fixation and soil fertility currently remains subject to much discussion, and

cannot be properly assessed due to the lack of accurate and sufficient data available (Stewart, 1969; Murphy, 1975).

There is a substantial amount of literature on the usage of symbiotic N-fixing bacteria in agriculture, whereas relatively less is known about the importance of free-living diazotrophs in similar environments (DeLuca *et al.*, 1996). Our understanding of these microorganisms is still extremely limited – whether regarding their identification or the physiological factors that control their activity. These major gaps are primarily ascribed to the methodological challenges in studying these N-fixers (Angel *et al.*, 2018). Many free-living diazotrophs are defiant to laboratory cultivation, whereas *in situ* rates of N_2 fixation by these microorganisms have yet to be measured with absolute certainty (Stewart, 1969; Hsu and Buckley, 2009).

1.5 Nitrogenase

The only currently known mechanism for BNF involves the nitrogenase enzyme. Nitrogenases are complex metalloenzymes with a highly conserved structure and mechanistic attributes that catalyse the reduction of N_2 to NH_3 (Eq. 1.1). These enzymes comprise two metalloprotein components and are referred to based their metal composition (Dixon and Kahn, 2004). The most common, and most comprehensively studied form of nitrogenase contain the molybdenum-iron (MoFe) and iron (Fe) proteins (Franche *et al.*, 2009). The larger 220,000 Da MoFe-protein tetramer, or Component I, contains the substrate-reducing enzyme catalytic site. Component II's dimeric Fe-protein of 68,000 Da is formed by identical subunits, and operates as the energy-dependent electron donor (Rees and Howard, 2000; Franche *et al.*, 2009). Alternative nitrogenases homologous to this system also exist, with Fe and/or vanadium (V) replacing the molybdenum (Mo) (Bishop and Joerger, 1990).

Each tetrameric MoFe protein further consists of two unique copies of metalloclusters (Fig. 1.5): 1) the FeMo-cofactor (FeMo-co M-cluster), and 2) the [8Fe–7S] P-cluster. The foremost subunit is likely to be the substrate reduction site, while the latter is understood to be an intermediate in the transport of electrons between FeMo-co and the Fe protein. A third subunit is also present, coordinated to the smaller Fe protein. This [4Fe–4S] F-cluster links together the two subunits of the dimer; but the Fe-S group here is surface-exposed, and thereby associates with the extremely oxygen (O_2) sensitive attribute of nitrogenase (Dixon and Kahn, 2004; Seefeldt *et al.*, 2004; Rubio and Ludden, 2008; Franche *et al.*, 2009; Hoffman *et al.*, 2014).

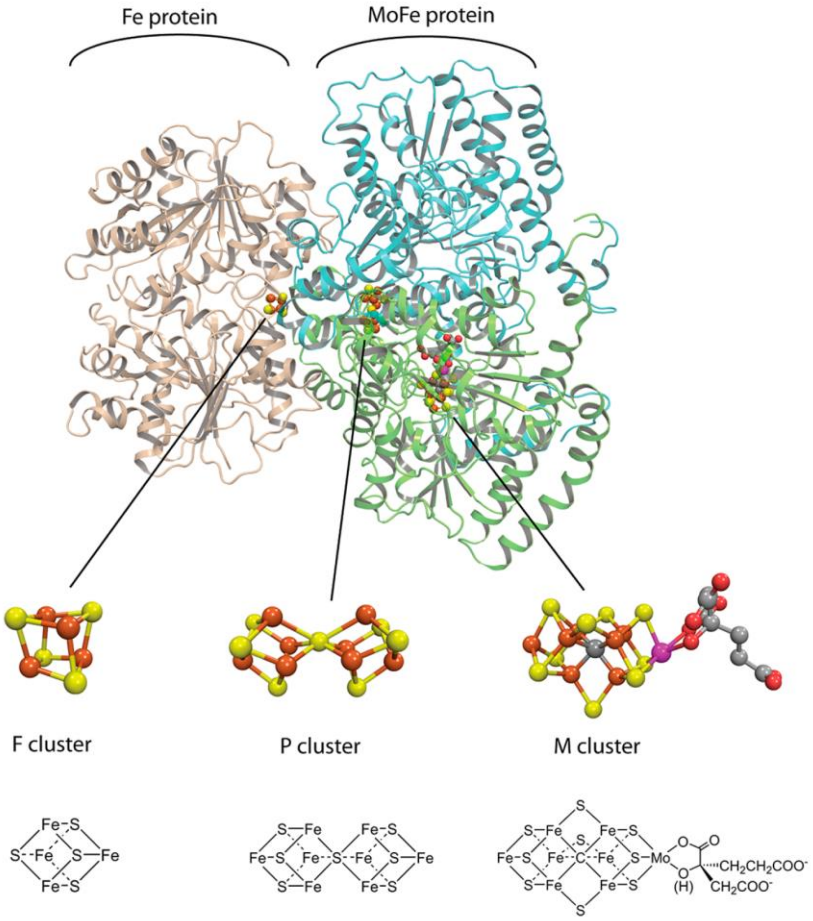


Figure 1.5. The structure of a molybdenum nitrogenase. The MoFe-protein subunits are shown in green and cyan, while the Fe-protein dimer is shown in light brown. Below are the molecular models and chemical structure of the [4Fe–4S] F-cluster, the [8Fe–7S] P-cluster, and the FeMo-co M-cluster. (Taken from Hoffman *et al.*, 2014).

BNF follows a mechanism catalysed by the nitrogenase enzyme. The Mo-dependent nitrogenase turnover cycle first requires electron donors to carry out the reduction of the Fe-protein. Once completed, single electrons are then transferred from the Fe-protein to the catalytic MoFe-protein; a step highly dependent upon ATP hydrolysis. Each electron transfer requires the two structural proteins to undergo a complex formation, after which is separated. Apart from energetic requirements, another role for ATP and its hydrolysis is to assure unidirectionality of the electron transfer; the input of ATP also prevents the return of the electron to the Fe-protein after its delivery to the MoFe-protein (Fisher and Newton, 2002). Finally, an internal electron transfer has to occur in the MoFe-protein, from the P-cluster to the M-cluster or substrate-binding site (Dixon and Kahn, 2004). The biological reduction of N_2 to NH_3 requires the cycle to keep

recurring for a minimum of 8 rounds, as each single electron transfer is coupled to 2 molecules of hydrolysed ATP (See Eq. 1.1; Duval *et al.*, 2013).

1.5.1 The *nif* gene

Nif genes are the key structural gene for nitrogenase, possessed by diazotrophs. Found in both symbiotic and free-living nitrogen-fixing bacteria, the genes which are essential for nitrogenase biosynthesis, electron transfer and regulation all fall into the *nif* regulon. Three genes – *nifD*, *nifK* and *nifH* – encode the structural constituents of the Mo nitrogenase enzyme complex. The MoFe-protein subunits are encoded by *nifDK*, while *nifH* is associated with the Fe-protein (Halbleib and Ludden, 2000; Rubio and Ludden, 2008). The latter gene possesses sites for ATP binding, and engage and disengage with the *nifDK* components amidst each electron transfer cycle (Rubio and Ludden, 2008). The O_2 sensitivity of nitrogenase for BNF is also reflected in its genetic expression, as the transcriptional regulation of *nif* genes are strictly monitored in response to external O_2 concentrations. (Dixon and Kahn, 2004).

Additional focus has been on the *nifH* gene as the molecular marker in identifying potential N-fixing microorganisms in a variety of natural environments (Zehr *et al.*, 2003; Bürgmann *et al.*, 2004; Gaby and Buckley, 2014). This is in part because *nifH* is better conserved than others, and therefore are amenable to molecular studies. Chien and Zinder (1994) divided the phylogeny of the *nifH* gene and its derivatives into four main clusters. Broadly, clusters I and III define the typical MoFe-nitrogenase, while other forms of nitrogenase that possess an alternative active site metal make up cluster II. Affiliates of cluster IV are highly divergent *nifH* paralogs or are microorganisms which contain more than one copy of nitrogenase genes; this cluster also involves gene products exclusively from methanogens (Chien and Zinder, 1994; Zehr *et al.*, 2003; Gaby *et al.*, 2018). Thus, the more conserved nature of *nifH* has allowed for its use as a biological tracker, where substantial insights have been attained on the microbial drivers of N_2 fixation.

1.5.2 Overcoming the effects of oxygen

As the protein components of nitrogenase are very O_2 sensitive, diazotrophs have evolved an array of physiological strategies to shield the enzyme from this gas. Spatial or temporal compartmentation of the enzyme, consumption of excess O_2 through respiration, or the simple

avoidance of O_2 through anaerobic growth are some tactics that have been developed and personalised to suit individual N-fixing species. Thus, the irreversible deactivation of nitrogenase by O_2 also inflicts sizeable physiological restrictions on diazotrophic activity as there is an overriding duty to protect nitrogenase from O_2 damage (Dixon and Kahn, 2004).

1.6 The distribution of organic N in soil

Large reservoirs of N are held in soils, with at least 90 % of the total N arising in organic forms (Senwo and Tabatabai, 1998; Friedel and Scheller, 2002). Through its impacts on nutrient availability and microbial activities, soil organic N plays an integral role in promoting plant growth and soil fertility (Kelley and Stevenson, 1995). Whether deriving from chemical fertilisers or natural sources, fixed N in the soil is acquired by plant roots and is ultimately utilised in the biosynthesis of biological molecules (Brill, 1977). The importance of soil organic N to the biological world is demonstrated by the prevalence of a broad series of N forms available in the soil – varying from amino acids and amino sugars, to the numerous heterocyclic polymers found in genetic material (Fig. 1.6; Bol *et al.*, 2004).

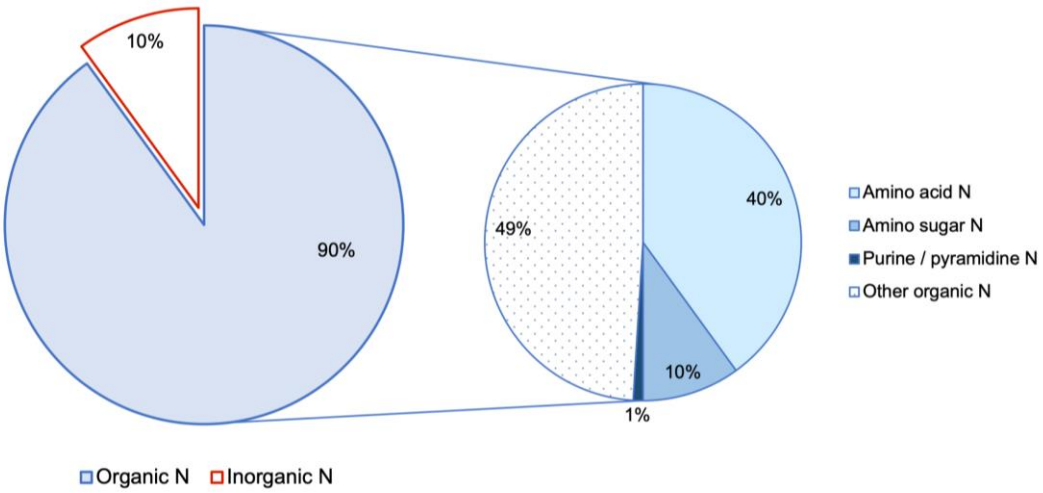


Figure 1.6. The soil N pool, and the corresponding proportions of soil organic N-containing compounds. Proteinaceous N materials including amino acids, proteins and peptides form the largest organic N fraction. Amino sugars, or amino sugar-containing biopolymers make up 3–10 %, whilst purine and pyrimidine derivatives are part of the various heterocyclic N compounds found in less than 1 % of SOM. (Data from Kuzyakov, 1997).

1.6.1 Amino acids and their assimilation

Proteins are composed of amino acids (AA) – all of which consists of at least one atom of N. Hydrolysis studies on surface soil show that AAs represent the largest proportion of organic nitrogenous products, reporting values between 20–60 % (Kuzyakov, 1997; Schulten and Schnitzer, 1998; Senwo and Tabatabai, 1998; Bol *et al.*, 2004). AAs are the basic building blocks of proteins, with 20 commonly occurring proteinogenic AAs abundant in biological systems operating as the proteins' functional units. In general, the canonical structure of AAs consists of an amino group and a carboxyl group at the α-carbon (Fig. 1.7). The key functional groups of an AA can go through condensation reactions with those of another to establish a peptide bond. These bonds allow for the formation of AA oligomers and polymers (i.e. peptides and proteins) vital to the survival of all living cells. Thus, a typical protein might be made up of a couple hundred AA units. Aside from being important sources of N for plants, AAs also hold various other roles: serving as energy sources for microorganisms, and substrates for enzymes in the turnover of N in soils (Senwo and Tabatabai, 1998; Moe, 2013).

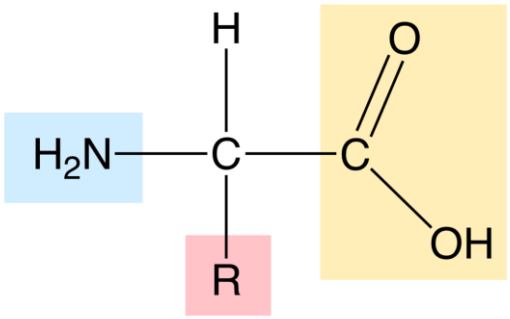


Figure 1.7. General AA structure, with the amino group in blue; the carboxyl group in yellow; and the R-side chain in red.

Not only do they embody the largest identifiable proportion of soil organic N, AAs are also crucial components in the assimilation and mineralization of N in soil. Kuzyakov (1997) proposed that organic N travels through the AA pool in the course of its diagenesis, while inorganic N within the soil travels through this pool during its assimilation into organic N compounds. Although only a restricted group of prokaryotes are able to reduce N_2 to NH_3, the latter form of N may be assimilated by most microorganisms for use in biosynthetic pathways. N assimilation incorporates NH_3 into AAs primarily through the N-transporting AAs, glutamate and glutamine (Miflin and Lea, 1976b; Schulten and Schnitzer, 1998). However, little

information is available on the AA pathway of N assimilation in regard to N_2 fixation. Further knowledge in this area will aid both our mechanistic and microbial understanding of this intricate process.

1.7 Methods for quantifying N_2 fixation

There are several explanations as to why the quantification of BNF is crucial in many aspects of this research. Without doubt, such measurements allow the proper assessment of diazotrophic activity and their ability to effectively fix N_2. In-depth evaluations of the physiology and functional constraints of these microorganisms can also be made as numerical values help to establish whether their full potential has been achieved. Furthermore, tracing the input of fixed N by diazotrophs can also influence soil management and ecological considerations – leading to the instigation of more sustainable agricultural practices and an enhanced understanding of the relative contribution of BNF to the N cycle (Peoples *et al.*, 1989).

While there is no one 'correct' way of quantifying N_2 fixation, each method possesses its own unique set of advantages and drawbacks to be carefully considered depending upon the user's individual objectives. The following section details important features of commonly used techniques for measurements of N_2 fixation, including the acetylene reduction assay, molecular genetics, and ^{15}N-stable isotope labelling. Finally, a shift towards compound-specific ^{15}N-stable isotope probing is suggested for obtaining measurements with higher sensitivity and accuracy.

1.7.1 The acetylene reduction assay

Historically, the acetylene reduction assay (ARA) has largely been the method used for determining BNF rates (Hardy *et al.*, 1973). This technique was made possible through the discovery that, in addition to N_2, nitrogenase also reduces acetylene (C_2H_2) to ethylene (C_2H_4). As C_2H_4 is easy to measure by gas chromatography, it was therefore proposed that the rate of C_2H_2 reduction be employed as an index of the rate of N_2 fixation (Stewart *et al.*, 1967). To rationalize for the greater amount of electrons required per one mole of fixed N_2, a stoichiometric conversion ratio of 3 : 1 is often used to extrapolate N_2 fixation rates (Eq. 1.3; Wilson *et al.*, 2012). In other words, the reduction of C_2H_2 to C_2H_4 should occur 3 times faster than the reduction of N_2 to NH_3 (Hardy *et al.*, 1968). Development of the ARA has allowed quicker determinations of

nitrogenase activity which was able to be administered not only to excised root nodules, but also soil cores, marine extracts and bacterial cultures (Francis and Alexander, 1972; Hardy *et al.*, 1973; Murphy, 1975; Montoya *et al.*, 1996; Wilson *et al.*, 2012).

$$N_2 + 6e^- + 6H^+ \xrightarrow{\text{nitrogenase}} 2NH_3$$

$$C_2H_2 + 2e^- + 2H^+ \xrightarrow{\text{nitrogenase}} C_2H_4$$

Equation 1.3. The quantitative relationship between two nitrogenase reductions. In the conversion of acetylene production into N_2 fixation rates, the frequently cited theoretical ratio of 3 : 1 stems from the two electrons/H^+ ions that are needed in the reduction of C_2H_2 to C_2H_4, whereas six electrons are required to reduce N_2 to $2NH_3$.

The ARA is often described in literature as a sensitive, practical and relatively inexpensive technique – but it does not come without limitations. A key advantage of this method is that it requires comparatively simple experimental instrumentation and produces rapid results for analysis (Montoya *et al.*, 1996; Wilson *et al.*, 2012). Nonetheless, the data analysis itself is often not so straightforward since the method only assays N_2 fixation through indirect means. The C_2H_2-to-N_2 ratio is merely a theoretical estimate, and values can deviate considerably in practice depending on a variety of factors (Nohrstedt, 1983; Carlsson and Huss-Danell, 2003). Direct calibration of the ARA with other techniques is therefore recommended for higher reliability (Montoya *et al.*, 1996).

It is also important to point out that the detection of nitrogenase activity through the ARA may sometimes be inadequate due to indirect effects posed by C_2H_2 on diazotrophic activity. As C_2H_4 is also an active hormone for plant growth, certain microorganisms can benefit from using it as a C source for microbial metabolism. Long-term incubations become complicated by this effect, leading to an underestimation of actual N_2 fixation (Dart and Day, 1971; Zechmeister-Boltenstern and Smith, 1998). C_2H_2 reduction is only able to provide instantaneous measurements of nitrogenase activity over short periods of time – making the technique a poor choice for looking at prolonged temporal changes in N-fixing activity (Peoples *et al.*, 1989; Carlsson and Huss-Danell, 2003).

Lastly, the ARA is known to have inhibitory effects on particular diazotrophs. These C_2H_2-sensitive microorganisms include methanotrophs and methanogens of the Archaea domain, as

well as other nitrifiers and sulphate reducers (Sprott *et al.*, 1982; Schink, 1985). If these N-fixers have anything to do with diazotrophy in the studied system, ARA may arrive at inaccurate estimates (Warren *et al.*, 2017). Experiments by Trinick (1980) on the inhibitory effect of the ARA also reported that repeated exposures of plants to C_2H_2 on consecutive days adversely affected their N metabolism and caused declines in nitrogenase activity (Fig. 1.8).

All in all, the ARA is an indirect assay of N_2 fixation, depending on the reduction of C_2H_2 as an analogue of N_2, and therefore may not be accurately compared (Montoya *et al.*, 1996). The multitude of technical problems summarised only allows for the fairly limited application of ARA to quantify N_2 fixation, or for use in combination with other methods.

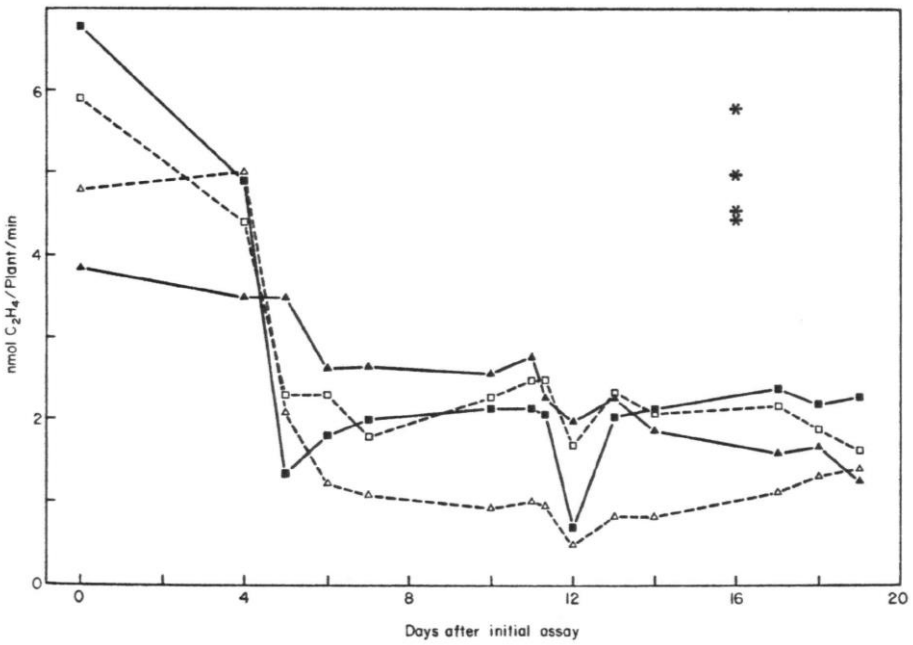


Figure 1.8. Inhibitory effect of successive C_2H_2 reduction assays on four like-sized plants over 19 days. Air was flushed through the tubes to eliminate residual C_2H_2 and C_2H_4. Asterisks (*) on day 16 indicate the rate of C_2H_2 reduction by control samples, i.e. those that had not formerly been exposed to C_2H_2. (Trinick, 1980).

1.7.2 Molecular genetic techniques

Molecular approaches like metagenomics and genetic fingerprinting do not directly quantify the rate of N_2 fixation but serves as important fingerprinting tools for identifying diazotroph communities. By targeting nitrogenase functional genes, some uncertainties regarding the role of diazotrophs in N_2 fixation can be clarified through an enhanced understanding of their functional gene dynamics (Levy-Booth *et al.*, 2014). The techniques provide a practical way of discovering

and classifying uncultivated N-fixing microbes, which can be associated to soil properties and N process rates (Fig. 1.9; Zehr *et al.*, 2003; Levy-Booth *et al.*, 2014). Hsu and Buckley (2009) also revealed that N_2 fixation rates showed a positive, though saturating, reaction to increased N-fixer diversity (Fig. 1.10a). Thus, the diazotroph community structure may show greater insights on fixation rates than lone soil characteristic measurements.

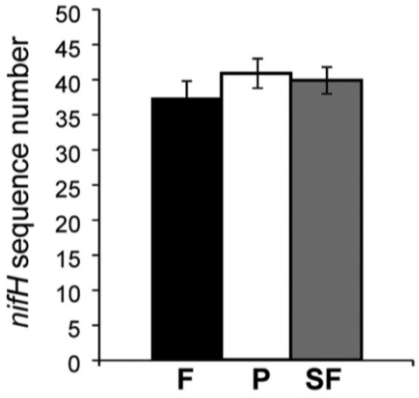


Figure 1.9. Number of unique *nifH* genes in response to changes in land-use in the Amazon. Different land-use treatments include: primary forest (F), pasture (P), and secondary forest (SF). (Mirza *et al.*, 2014).

Molecular analyses of N_2 fixation employ the nitrogenase *nifH* gene as a marker of choice (Zehr *et al.*, 2003; Gaby *et al.*, 2018). Throughout the *nif* operon, *nifH* is recognised as the most conserved gene; accordingly, it has been at the focus of investigations on the genetic abundances and distribution of diazotrophs across various settings (Orr *et al.*, 2011; Angel *et al.*, 2018). The process, however, must be conducted with some caution as non-cultivated N-fixers can presently only be identified based on detection of the *nifH* gene. Incongruences between the phylogeny of the gene and that of the organism, alongside the lack of consideration for alternative nitrogenase systems, render information obtained from *nifH* genetic sequences alone to be rather restrictive (Bürgmann *et al.*, 2004; Buckley *et al.*, 2008).

Various methods with differing benefits and limitations exist for the characterisation of diazotrophs and their activity – although polymerase chain reaction (PCR)-based methods have been popular amongst many studies (Bürgmann *et al.*, 2004; Morales *et al.*, 2010; Mirza *et al.*, 2014; Gaby *et al.*, 2018). This strategy usually involves the total extraction of deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) from a sample, which is later applied as a template for the identification of microorganisms. Amplified PCR products are then predominantly analysed via genetic fingerprinting or in combination with other quantification techniques (Rastogi and

Sani, 2011). PCR-based methods have contributed significantly to the understanding of functional N-fixing genes within soil, and are known to be very reproducible while also delivering taxonomic information and microbial abundances of high resolutions (Levy-Booth *et al.*, 2014).

Nevertheless, DNA-based molecular analysis techniques remain liable to methodological bias. Microbial diversity data can be visualized through a great number of ways and deciding the most suitable and informative representations can be challenging. Also, a reference database with a higher level of functionality and coverage is still to be developed for the *nifH* gene (Gaby *et al.*, 2018). While universal primers for the functional gene have been designed to specifically target the regions encoding conserved AA sequences, the protocol development and selection is also subject to inaccuracies. Primers are either highly degenerate to avoid significant variability in the DNA sequence or focus on smaller subsets of diazotrophs to evade high degeneracy. Even so, not all *nifH* genes will be amplified with primer sets of low-degeneracy; while the combined use of highly degenerate primers alongside amplification conditions of low-stringency are prone to generate subjective results and may lessen the suitability of the protocol for more quantitative and detailed studies (Bürgmann *et al.*, 2004; Levy-Booth *et al.*, 2014).

Moreover, it must also be noted that the presence of *nifH* genes do not always denote an active N-fixing community (Fig. 10b; Wertz *et al.*, 2012). Only the potential activity is able to be resolved when soil samples undergo nucleic extraction and amplification, as there is no way to distinguish between active, dormant or dead DNA sources (Levy-Booth *et al.*, 2014). Nitrogenase functional genes are often sporadically distributed, to the extent that phylogenetic connections may not always infer N-fixing capabilities (Zehr *et al.*, 2003). Tight cellular regulation due to the demanding energetic requirements of N_2 fixation render the presence of *nifH* genes – or even their transcripts – alone to frequently reflect an inaccurate representation of the functioning diazotroph community and their rates of N_2 fixation (Angel *et al.*, 2018).

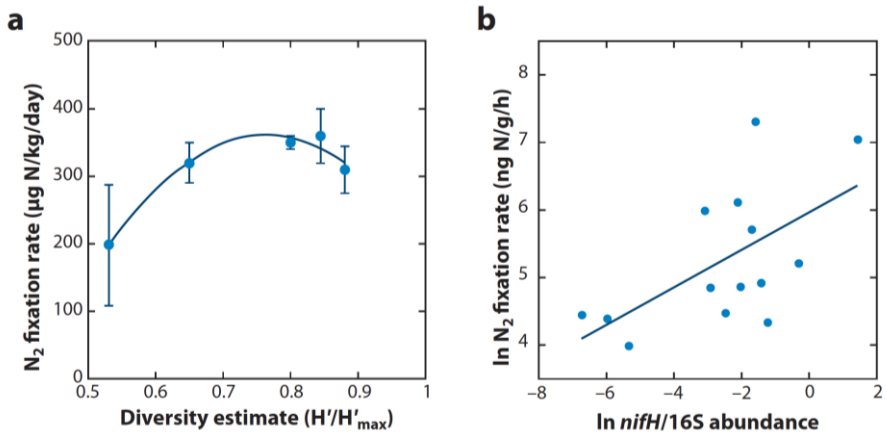


Figure 1.10. Relationships between N_2 fixation rates and the diazotroph community structure: **a)** saturating relationship between the diversity of N-fixers and the rate of N_2 fixation, in a soil which received different agricultural treatments (data from Hsu and Buckley, 2009); and **b)** relationship between the relative abundance of the *nifH* gene and N_2 fixation rates, in a tropical rainforest leaf litter ($r^2 = 0.35$; data from Reed *et al.*, 2010). (Figure taken from Reed *et al.*, 2011).

1.7.3 ^{15}N-stable isotope labelling

Recent studies have proceeded to monitor N_2 fixation by incorporating ^{15}N-stable isotope tracers. This method exploits the difference in atomic mass which makes it analytically possible to distinguish stable isotopes from one another; thus, making it possible to trace their metabolic fate when introduced into a system (Wilkinson, 2018). Samples are given a unique isotopic signature via enrichment with a ^{15}N-labelled substance or gas over time, and the applied ^{15}N is subsequently assimilated by effective diazotrophs into their biomass (Montoya *et al.*, 1996). The isotopic enrichment accumulated across time is then translated into a measure of the N-fixing activity (Angel *et al.*, 2018).

Isotope measurements are traditionally taken from the entire sample – i.e. the bulk soil; however, drawbacks arising from the complex and heterogeneous nature of soil organic N have held back detailed investigations into the activity of diazotrophs. In the extraction of ^{15}N-labelled bulk soils, the measurement of the ^{15}N : ^{14}N ratio, or δ^{15}N value, is not only contingent on the diazotrophic activity but also the amount of background N within the sample as well. Isotopic signals from effective free-living N-fixers are mixed into the large pool of passive N accumulated in soil organic matter (SOM), hence typically causing tracer dilution and lowered sensitivity such that the ^{15}N-isotopic incorporation can barely be seen (Angel *et al.*, 2018). Bulk ^{15}N analyses also fail to

provide useful insights into the assimilation pathways of microorganisms throughout the N_2 fixation process. Thus, from a critical standpoint, this method of analysis allows for the applied ^{15}N-substrate to merely function as a physical tracer – as opposed to an actual biochemical tracer of the system (Charteris *et al.*, 2016).

1.7.3.1 ^{15}N-DNA/RNA-SIP

An elegant approach which examines diazotrophic activity in further detail is nucleic acid-based ^{15}N-stable isotope probing (^{15}N-SIP). The soil sample is incubated with an added ^{15}N-substrate and ^{15}N-labelled DNA or RNA are separated from their unenriched counterparts by pairing the technique with caesium chloride (CsCl) density gradient centrifugation (Meselson and Stahl, 1958; Radajewski *et al.*, 2003). This allows active free-living N-fixers in a system to be identified without using the *nifH* marker gene; the isolated enriched substrates can subsequently be utilised as a template for quantitative PCR (Cadisch *et al.*, 2005). ^{15}N-DNA-SIP or ^{15}N-RNA-SIP offers a window into the performance of functioning participants in the BNF process, where the taxonomic profile of non-cultivated diazotrophs may also be established (Buckley *et al.*, 2007, 2008).

However, the method of DNA/RNA-based SIP possesses significant limitations. One is the need for highly elevated final nucleic acid ^{15}N-enrichments – ideally >50 % (Cadisch *et al.*, 2005). The separation of heavy and light labelled-DNA/RNA by employing density gradient centrifugation relies on the large availability of highly enriched ^{15}N-DNA/RNA in order to visualise distinct bands (Radajewski *et al.*, 2003). Consequently, as cells undergo assimilation from both native and enriched sources, dilution of the isotopic label within the receiving community results in the necessity to add ^{15}N-labelled substrates at substantially higher concentrations. Lower growth rates in soil generate another setback with nucleic acid-SIP experiments as it may entail extended incubation times to enable adequate labelling. Longer incubations also increase the potential for cross-feeding and trophic cascades as the isotopic label transfers into nucleic acids from non-targeted functional groups (Buckley *et al.*, 2007, 2008).

As opposed to ^{15}N-DNA-SIP, RNA-SIP experiments have shown more sensitivity and faster tracer incorporation; however, evidence for the cycling of N within the soil community is still unavailable. Studies have revealed that the assessment of stable isotope assimilation into RNA has several benefits over DNA including faster incorporation and labelling which is free of cell

replication (Manefield *et al.*, 2002; Cadisch *et al.*, 2005; Angel *et al.*, 2018). Nevertheless, another integral part of nucleic acid-SIP approaches is the use of PCR primers for the theoretical validation of enriched DNA or RNA – which, as previously mentioned, is a technique subject to its own limitations of methodological biases. Additionally, although helpful in identifying active diazotrophs, nucleic acid ^{15}N-SIP approaches do not present insights regarding the fate of applied ^{15}N within the soil biomass (Charteris *et al.*, 2016).

1.7.3.2 Compound-specific ^{15}N-stable isotope probing

A promising technique within the realm of SIP that can meet such requirements is compound-specific ^{15}N-SIP using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). As implied by the name, the basic components of the instrument include a GC unit for compound separation and an IRMS unit for isotopic analysis. The two parts are coupled by a combustion reactor, which receives N compounds eluting from the GC column and submits them to the IRMS for information on the N isotopes (Leeuwen *et al.*, 2014).

The great benefit of this technique lies in its sensitivity and specificity. This allows for the application of ^{15}N-substrates at environmentally relevant concentrations, and for enrichments at appropriately lower levels to be traced through an array of ecosystems. While IRMS has been extensively used due to the method's precision and ability to analyse small samples, only a small number of researchers have exploited the far more superior potential precision of GC-C-IRMS – ranging between 0.5–2.0 ‰ or 0.0002–0.0008 atom % (Merritt and Hayes, 1994; Metges *et al.*, 1996). Of these studies, most are related to the physiology of mammals and their trophic levels (Petzke *et al.*, 1997; Dänicke *et al.*, 1999; Kendall *et al.*, 2017); while the remainder focuses on other research areas such as plant N uptake and their microbial associations (Sauheitl *et al.*, 2009; Molero *et al.*, 2011). Latterly, studies have come to focus on the cycling of organic N in soil and have consequently started to hint towards the prospect of ^{15}N-SIP using GC-C-IRMS as a hugely powerful tool for following the fate of applied ^{15}N into the soil biomass (Knowles *et al.*, 2010; Redmile-Gordon *et al.*, 2015).

The sensitivity of this approach is enhanced by specifically targeting AAs during analysis. As the main 'building blocks' of life, AAs represent a major portion of soil organic N and therefore take up a leading role in N transformation processes (Wanek *et al.*, 2010). Thus, by singling out AAs, compound-specific ^{15}N-SIP offers a highly sensitive approach as these compounds become

distinguishable from the thousands of proteins present within the sample; alongside revealing important characteristics regarding the dynamics and assimilation pathways of N-substrates into the soil organic N pool (Charteris *et al.*, 2016).

Studies conducted by Charteris *et al.* (2016) highlighted that the amount of applied ^{15}N measurable in the total hydrolysable AA pool can be extended as a proxy for the proportion of applied ^{15}N assimilated by the soil microbial biomass and percentage ^{15}N-incorporations can be derived. Advantages of the compound-specific method are evident in Figure 1.11, where visible changes are almost immediately detectable in the $\delta^{15}N$ values of all hydrolysable AAs. Measurements were able to be translated into % incorporations of the applied ^{15}N-substrate, whereby differences in patterns could be related to relevant biosynthetic pathways (Fig. 1.11). Such evidence has been inaccessible via previous procedures, making compound-specific ^{15}N-SIP using GC-C-IRMS potentially applicable for studying N-cycling of any soil system and over a broad range of environments. This method can be applied to quantify N_2 fixation – where % ^{15}N-incorporation would be extended as the % of N fixed by active diazotrophs – and identify potential pathways of N assimilation accompanying the process.

Given its advantages, compound-specific ^{15}N-SIP has surprisingly been predominantly unexploited, and so, its entire scope of applications and potentials are yet to be fully realised. Challenges linked to compound-specific ^{15}N-SIP using GC-C-IRMS may be partly responsible for this (Charteris *et al.*, 2016). In the past few decades, the complexity and costliness of the method have generally discouraged its use. The application of the technique for ^{15}N analyses can make it somewhat temperamental when compared with those of ^{13}C. This is due to a variety of factors including the much lower abundance of N in organic molecules as opposed to C; the need for two atoms of N to produce each molecule of N_2 and required further reduction chemistry to effectively transform a N-containing molecule into N_2 for analysis; the ionisation efficiency of N_2 being only 70% relative to carbon dioxide (CO_2); and the profusion of N_2 in the air can cause small leaks in the system to be detrimental (Brand *et al.*, 1994; Brenna, 1994). Nonetheless, the lack of interest in compound-specific ^{15}N-SIP via GC-C-IRMS may also be due to inadequate awareness concerning its potential to examine soil N cycling, including N_2 fixation.

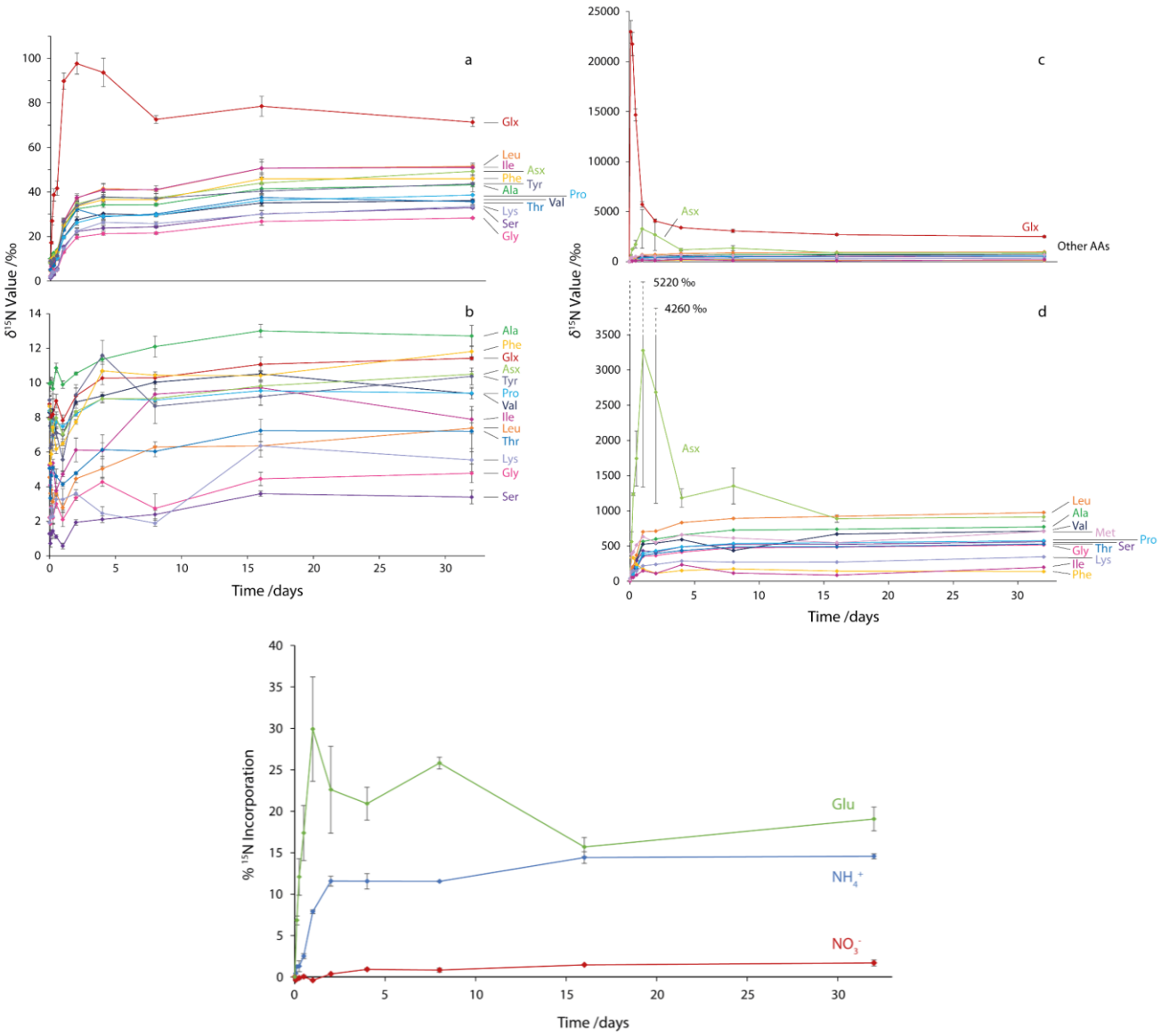


Figure 1.11. $\delta^{15}N$ values of individual hydrolysable AAs for soil incubation experiments over 32 days, and the corresponding % ^{15}N-incorporations. The four graphs above represent incubations involving applied: a) $^{15}[NH_4]^+$, b) $^{15}[NO_3]$, c) ^{15}N-Glu, including the applied ^{15}N-Glu and d) excluding the applied ^{15}N-Glu. Percentage of applied ^{15}N incorporations into the total hydrolysable AA pool for each substrate are shown in the graph below. (Taken from Charteris *et al.*, 2016).

1.8 Aims and objectives: why fix it?

What Fritz Haber could not foresee at the time of his breakthrough was the torrent of environmental consequences that was to come with the massive increase in NH_3 production. Distressing greenhouse gas levels, increases in water and air pollution, and irreparable damages to ecosystems are just a few examples of environmental concerns which have manifested. The world population has increased ten-fold over the centuries, reaching 7 billion in 2011, and is projected to reach 9.8 billion by 2050 (UN, 2017). The exponential growth of the global population since the 20th Century has, without doubt, been largely supported by elevated N-input into agricultural soils – primarily in the form of chemical N-fertiliser. While important benefits of N_2 fixation via the Haber-Bosch has fed the stomachs of billions, food security must also be balanced with the effective use of N that minimises damaging environmental impacts.

Thus, this is where the microbial N cycle and BNF plays an essential role. Fixed N by this route is utilised in-situ, and is therefore less prone to leaching (Dixon and Kahn, 2004). Pressures arising from the limited availability of arable land, population growth, and aggravating climate change has sparked further interest in the possibility of BNF as an important and sustainable solution for agricultural systems (Hardy and Havelka, 1975; Dixon and Kahn, 2004). Cascading environmental and economic constraints will presumably prohibit magnifying the use of chemical N fertiliser appropriately; so BNF – as a solely microbiological process – must be manipulated more effectively.

Significant limitations in our understanding of BNF and the capabilities of these N-fixers still remain. While the molecular biology of N_2 fixation is rather well-studied, ecological information on symbiotic, and especially free-living, N-fixing systems are substantially weaker (Vitousek *et al.*, 2002). If BNF is to provide a reliable and renewable resource for future generations, it is necessary to assess the effectiveness of the process by accurately quantifying N fixed by various diazotrophs in a range of environments. The lack of understanding as to what makes some systems fix N better than others must be further explored. Likewise, the distribution and pathways of N in the soil pool in regards to BNF, as well as the possibilities of improving BNF inputs also need to be properly assessed (Peoples *et al.*, 1995).

1.8.1 Can we fix it?

This research seeks to address these questions by developing a novel ^{15}N-SIP method for quantifying N_2 fixation in soils, by creating an assay with greater sensitivity and specificity than is currently available. Compound-specific AA analysis will allow assessment of the uptake of applied $^{15}N_2$ gas by the soil microbial biomass; the rate and magnitude of assimilation by diazotrophs will be measurable through the ^{15}N-content of newly synthesized AAs as determined by GC-C-IRMS. This new technique will aim to: i) provide greater specificity compared to existing methods, and ii) contribute previously unobtainable quantitative information on the capture and flow of N in agricultural and semi-natural soils. Although the ultimate interest lies in free-living N-fixers, initial work will be done on symbiotic N-fixers to develop and test the method at hand. The refined process will then be adapted and applied to a variety of free-living diazotrophs from different habitats and ecosystems. Ensuing knowledge on the biological input of N and an improved understanding of its molecular mechanism can lead to a more effective development of targets for its ultimate use.

2 Materials and experimental methods

2.1 Samples

Sampling was carried out from early to mid-2018 around Devon, in southwest England. The types of plant and soil samples collected were selected on the premises of existing studies regarding N-fixers in order to establish a feasible baseline for the method development and subsequent results. Once acquired, samples were stored at 4 °C to slow-down microbial activity until the start of laboratory incubation experiments.

2.2 Incubations

Incubations were carried out in 30 mL glass serum vials, where each soil or plant sample occupied approximately two-thirds of the vial volume (around 7–7.5 g depending on the type of sample). A methylene blue anaerobic indicator strip (Anaerotest®; Merck, Darmstadt, Germany) was also placed inside the vial to monitor the oxygen content within the system. Vials were then tightly sealed with grey butyl rubber stoppers and aluminium crimp seals. A plastic gas tight syringe (10 mL), fitted with a needle, was used to supplement 10% of the vial headspace ^{15}N-labelled nitrogen gas (3 mL $^{15}N_2$, 98 % atom; CK Isotopes, Leicestershire, UK) immediately after the system was closed off.

Carbon dioxide (CO_2) traps were created (360 mm length x 7 mm diameter, 0.45 cm^3 average volume) for each incubation vial using polyvinyl chloride (PVC) tubing fitted with a combi-stopper on one end and a disposable hypodermic needle on the other. Inside each tubing, a lightly ground up sodium hydroxide pellet (NaOH; Fisher Scientific, Loughborough, UK) was secured in between small wads of glass wool to absorb CO_2 released through soil respiration and prevent the system from turning anaerobic too rapidly. The needle end was inserted through the rubber stopper on the vial once $^{15}N_2$ gas had been introduced into the system (Fig. 2.1).

All incubations were conducted in triplicate. Vials were incubated in the dark at room temperature for the duration of the experiment. At specific time-points, the incubations were halted by submersion in liquid N_2 to immediately halt all biological activity. The samples were then stored at -20 °C until freeze-drying.

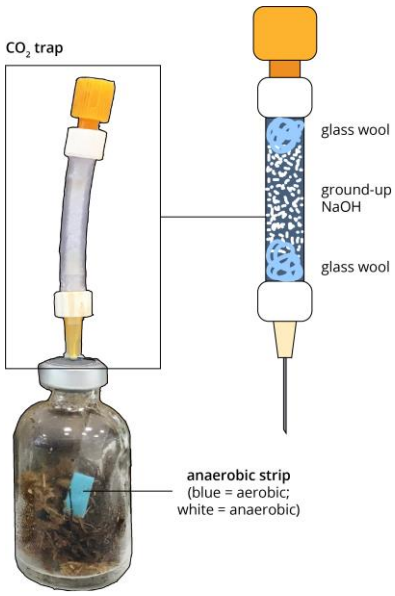


Figure 2.1. The incubation setup. The CO_2 trap contains ground-up NaOH held in-between wads of glass wool and the trap is attached to the vial via the needle end.

2.3 Hydrolysable amino acid analyses

All solvents used in this study were of HPLC grade and purchased from Rathburn Chemicals Ltd. (Walkerburn, UK). Double distilled water (DDW) was generated using a Bibby Aquatron water still. Derivatising agents – acetyl chloride, triethylamine, and acetic anhydride – were obtained from Sigma-Aldrich (Steinheim, Germany).

Glassware was all thoroughly cleaned with Decon 90 and furnaced at 450 °C for 4 h before use. To prevent contamination, all glassware and equipment in contact with ^{15}N-enriched samples were washed and used in isolation. A separate set of glassware and equipment was employed for control samples of natural abundance.

2.3.1 Preparation of amino acid standards

A solution of D,L-AA standards (1 mg mL^{-1} of each AA) was prepared in 0.1 M hydrochloric acid (HCl; reagent grade, Fisher Scientific) by the addition of 14 AA standards, of known δ^{15}N values. These AAs comprised of: alanine (Ala), valine (Val), glycine (Gly), leucine (Leu), isoleucine (Ile), threonine (Thr), serine (Ser), proline (Pro), aspartic acid (Asp), glutamic acid

(Glu), hydroxyproline (Hyp), phenylalanine (Phe), lysine (Lys), and tyrosine (Tyr) (Sigma-Aldrich). An internal standard (norleucine, Nle; Sigma-Aldrich) was also added to the mixture to allow quantification.

2.3.2 Sample preparation

Plant and soil samples were freeze-dried and finely ground using a mortar and pestle. For roots and fibrous peat material, scissors were used to cut the sample into small pieces, before being further homogenised until plant tissues largely assumed the consistency of flour.

2.3.3 Acid hydrolysis of plant and soil material

Norleucine (Nle; 800 μg mL^{-1} in 0.1 M HCl) was added as an internal standard (I.S.) to *ca.* 100–200 mg of finely ground soils or plant roots, which had been weighed into culture tubes. Hydrochloric acid (4 mL; 6 M) was added to each sample, before filling the headspace with oxygen-free N_2 gas. The tubes were then tightly sealed and heated at 100 °C for 24 h (Fig. 2.2). Once the samples were cooled, hydrolysates were separated by centrifugation (3500 rpm, 10 min). The supernatant was transferred to a 28 mL glass vial, and remaining residues washed twice with 2 mL 0.1 M HCl, centrifuged, as before, with the ensuing supernatants combined. Finally, the soil and root hydrolysates were dried under a stream of N_2 at 60 °C, and stored in 1 mL 0.1 M HCl at -20 °C.

Acid hydrolysis not only extracts both proteinaceous and free AAs, but also kills living microbial biomass (Roberts and Jones, 2008). The fairly rigorous conditions are required to cleave the peptide bonds between hydrophobic AA residues, such as Val, Leu and Ile. However, this also led to the deamination of asparagine (Asn) and glutamine (Gln) to Asp and Glu, respectively, as well as the total loss of cysteine (Cys) and tryptophan (Trp) (Fountoulakis and Lahm, 1998; Roberts and Jones, 2008). Hence, the N of Asp and the amino N of Asn are combined to produce the $\delta^{15}N$ value of Asx during analysis; while Glx is the mean $\delta^{15}N$ value of the Glu N and the Gln amino N. Hydrolysis needs to be carried out under an atmosphere of N_2 to minimise oxidative degradation of hydroxyl- and sulphur-containing AAs (Roberts and Jones, 2008). Certain AAs may also be partially destroyed by the technique: Thr (loss of *ca.* 5 %), Ser (loss of *ca.* 10 %) and Tyr (loss is dependent on the extent of trace impurities within the hydrolysing

agent) (Fountoulakis and Lahm, 1998). Particular AAs may also be overestimated – namely Ala, Gly, Glu and Lys – due to the possibility of hydrolysing AAs from non-proteinaceous sources (e.g. peptidoglycan) (Roberts and Jones, 2008). Nevertheless, the protocol is considered as the most reliable means for evaluating the total soil protein content, such that hydrolysable AA concentrations of a soil sample can therefore be rationally equated to the size of its protein pool (Roberts and Jones, 2008; Charteris *et al.*, 2016).

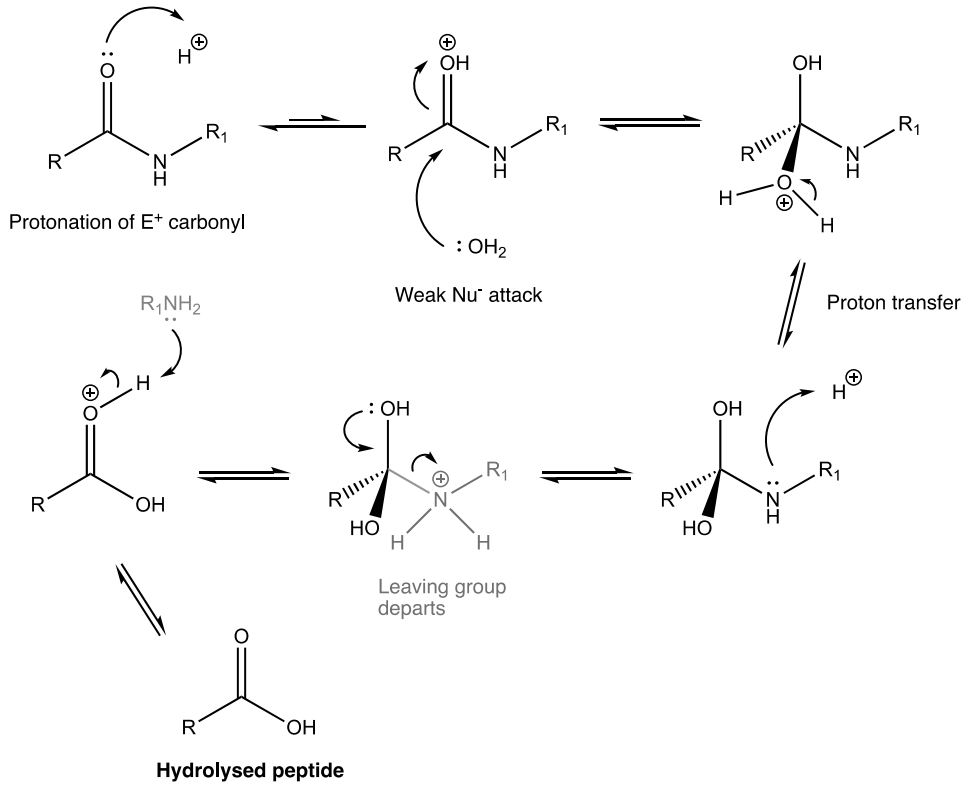


Figure 2.2. Mechanism for the extraction of AAs by acid hydrolysis. E^+represents electrophilic; Nu^- denotes nucleophilic.

2.3.4 Purification of extracted amino acids

AAs were isolated and purified by cation-exchange column chromatography using a Dowex 50WX8 ion-exchange resin (200-400 mesh; Acros Organics, Morris Plains, NJ, USA). To ensure that H^+ ions are occupying all cation-exchange sites, the resin was prepared by soaking overnight in 3M NaOH (Styring *et al.*, 2012). The liquid was then discarded and any excess NaOH removed by washing the resin with DDW. For each wash, the resin was first agitated and allowed to settle, before the water was poured away. This was repeated five times, or until the pH of the

wash tested neutral on pH paper (Fisherbrand pH Indicator Paper; Fisher Scientific). Finally, the washed resin was stored in 6M HCl for at least 12 h before use.

Approximately 1 mL of the prepared Dowex resin was placed in a flash column and washed again by passing through DDW (6 x 2 mL). The surface of the resin was not allowed to dry until very end of the column chromatography procedure. Soil and root hydrolysates previously stored in 1 mL 0.1 M HCl were then added to the column. Another 1 mL of 0.1 M HCl was mixed inside the sample vial to ensure that all AA hydrolysates were transferred to the column. Salts were eluted first with DDW (2 x 2 mL), and finally the AAs were eluted into clean culture tubes with 2 M ammonium hydroxide (2 x 2 mL NH_4OH; Fisher Scientific). The collected AA fraction was then blown to dryness under N_2 at 60 °C, before undergoing derivatization.

2.3.5 *N*-acetyl, *O*-isopropyl derivatisation of amino acids

AA *N*-acetyl, *O*-isopropyl (NAIP) ester derivatives were prepared based on established protocols (Corr *et al.*, 2007b). In the isopropylation of AAs, a mixture of isopropanol and acetyl chloride (4:1, v/v) was made by the dropwise addition of acetyl chloride (AC; puriss. p.a. grade) into an ice-cold vial of isopropanol (IPA). Then, 0.5 mL of this mixture was added to the hydrolysed AA fraction and heated at 100 °C for 1 h (Fig. 2.3). The reaction was quenched by placing the culture tubes in a freezer at -20 °C, followed by the removal of solvent remnants by evaporation under a gentle stream of N_2 at 40 °C. To ensure that all excess reagents are removed, the sample was re-dissolved in DCM (2 x 0.25 mL) and gently blown down.

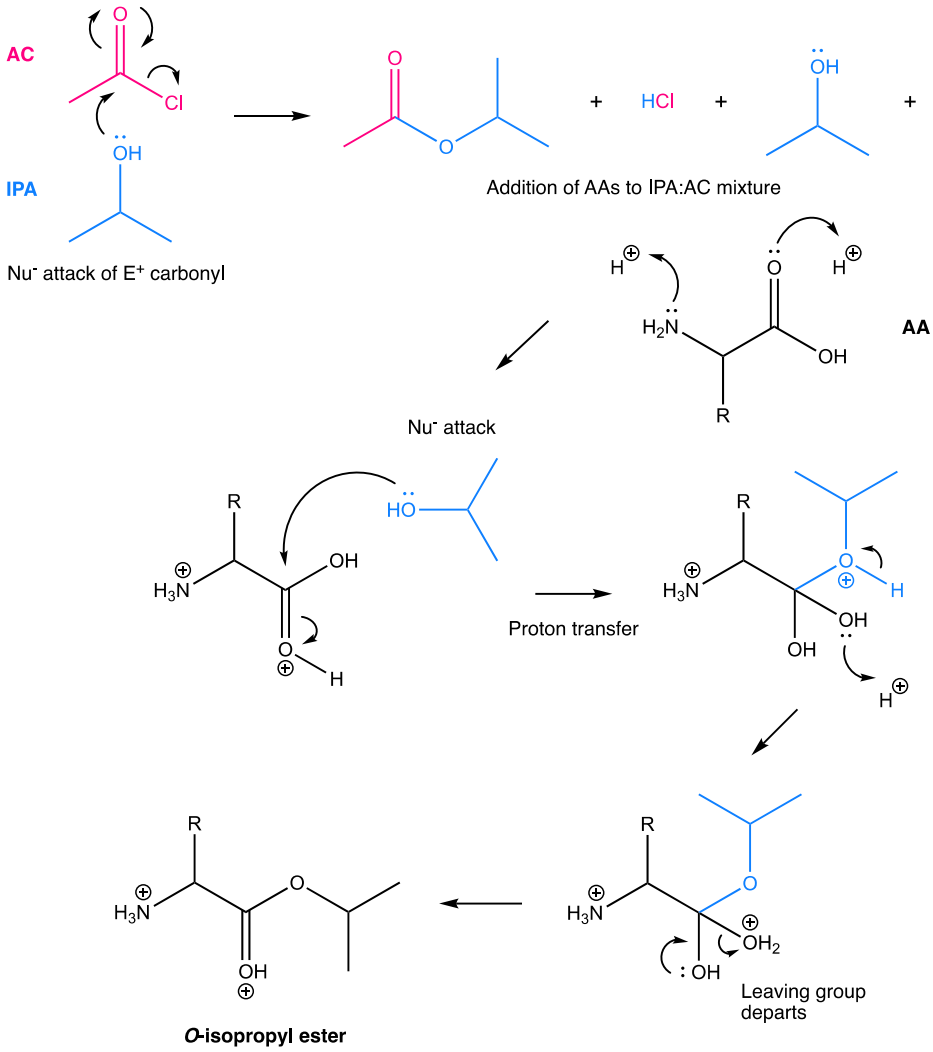


Figure 2.3. Mechanism for the *O*-isopropyl derivatization of AAs. The skeletal structure in blue represents isopropanol (IPA), while the structure in pink is acetyl chloride (AC).

Subsequently, AA isopropyl esters were then treated with 1 mL each of a freshly made 5 : 2 :1, v/v/v mixture of acetone, triethylamine (Et_3N; ≥ 99.50 % purity), and acetic anhydride (Ac_2O; ReagentPlus® grade). The culture tubes were sealed with PTFE and heated at 60 °C for 10 min (Fig. 2.4). A gentle stream of N_2 was used to evaporate the reagents at room temperature. The samples were then dissolved in 2 mL ethyl acetate, alongside the addition of 1 mL saturated NaCl solution. Vortex mixing was used to enforce phase separation, in which the organic layer was drawn off and dried under N_2 at room temperature. Any residual water was removed with successive aliquots of DCM (3 x 1 mL), getting blown to dryness each time under a gentle stream of N_2 in an ice bath. Finally, the AA NAIP ester derivatives were dissolved in ethyl acetate and stored at -20 °C until analysis.

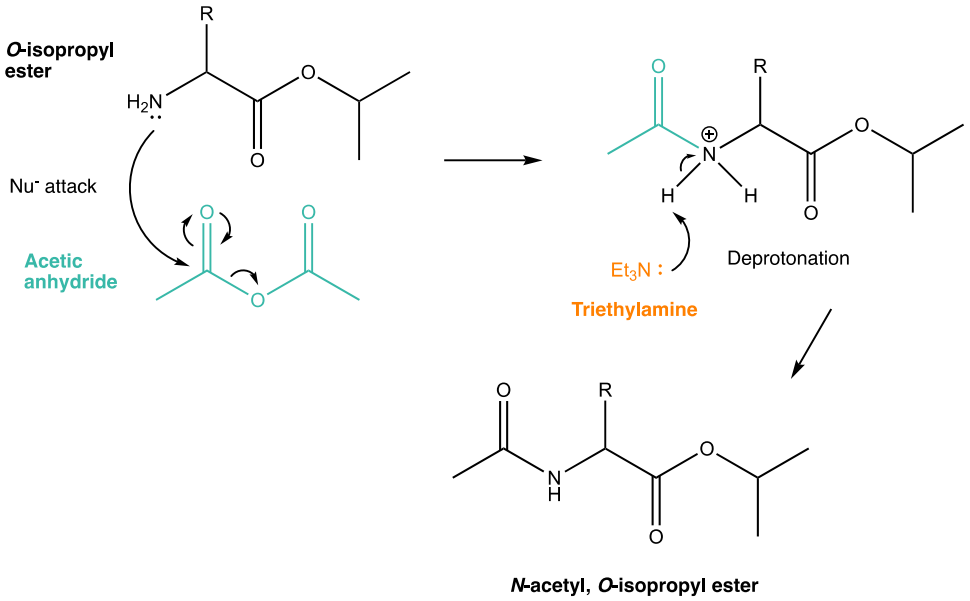


Figure 2.4. Mechanism for the *N*-acetylation of AAs; the final step of the AA derivatization protocol.

2.4 Instrumental analyses

2.4.1 Bulk soil $\delta^{15}N$ analyses

Bulk soil $\delta^{15}N$ analysis was carried out using a Flash 1112 elemental analyser (EA; Thermo Scientific, Milan, Italy) coupled under continuous flow to a DeltaPlus XP isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany). Finely ground soil or roots (*ca.* 9 mg) were weighed into tin capsules, prior to sample combustion and subsequent reduction over heated copper (Cu) wires within the EA. The resulting N_2 was transferred into the IRMS for determination of $\delta^{15}N$ values, where isotope ratios were calculated as $\delta^{15}N$ *vs.* atmospheric N_2.

2.4.2 GC-FID

An Agilent Technologies 7890B GC System fitted with a DB-35 column (35%-phenyl-methylpolysiloxane; mid-polarity, low bleed; 30 m x 0.32 mm internal diameter x 0.5 µm film thickness; Agilent Technologies, Santa Clara, CA, USA), and samples were introduced via on-column injection. Helium (He) was used as the carrier gas, with the instrument running under constant pressure. The oven temperature programme was held at an initial temperature of 70 °C for 2 min, before heating to 150 °C at 15 °C min^{-1}, then to 210 °C at 2 °C min^{-1}, and finally to 270

°C at 8 °C min^{-1}, where it was held for 10 min. A flame ionization detector (FID) was used to detect column eluent. AAs as their *N*-acetyl, *O*-isopropyl derivatives were identified by comparing their relative retention times with those of derivatised AA standards. Peaks were quantified by comparison with an I.S. (Nle) of known concentration.

2.4.3 GC-C-IRMS

The $\delta^{15}N$ values of individual AAs as their derivatised forms were determined by GC-C-IRMS using a ThermoFinnigan Delta^{Plus} XP isotope ratio mass spectrometer (Thermo Electron Corp., Waltham, MA, USA). The mass spectrometer was coupled with a ThermoFinnigan Trace 2000 gas chromatograph via a ThermoFinnigan Combustion III Interface. A DB-35 capillary column (30 m x 0.32 mm i.d., 0.50 μm film thickness; Agilent Technologies) was fitted in the GC to achieve the separation of the AAs. High purity copper (Cu) and nickel (Ni) wires (OEA Laboratories LTD, Callington, UK) made up the oxidation reactor which was maintained at 1030 °C, whereas the reduction reactor composed of only Cu wires was maintained at 650 °C. A GC Pal autosampler (CTC Analytics, Zwingen, Switzerland) was used to introduce the samples via a programmed temperature vapourising (PTV) injector (Thermo Electron). A He carrier gas was used at a flow of 1.4 mL min^{-1}. The oven temperature was programmed as follows: starting at 40 °C and held for 5 min, then heated to 120 °C at 15 °C min^{-1}, to 180 °C at 3 °C min^{-1}, to 210 °C at 1.5 °C min^{-1}, and finally to 270 °C at 5 °C min^{-1} and held for 1 min.

All the AA $\delta^{15}N$ values were determined in relation to a reference N_2 gas of known N isotopic composition. This monitoring gas was introduced via an open split straight into the ion source in four pulses, at the start and end of each run. AA peaks were identified by comparing the retention times with those of derivatised AA standards, and reported values for each sample are an average of duplicate $\delta^{15}N$ measurements. In order to ensure proper instrument function, the AA standard mixture of known $\delta^{15}N$ values was analysed between every three sample runs. Sample AA $\delta^{15}N$ values were only permitted when most of the AA standards bracketing the sample were within ±1 ‰ and the rest within ±1.5 ‰ throughout the course of the run. Data was acquired and processed using Isodat NT 3.0 software suite (Thermo Scientific). A typical GC-C-IRMS chromatogram of derivatised hydrolysable AAs in a soil sample is shown in Figure 2.5, including the signal intensity of ions for each mass-to-charge ratio (*m/z*) recorded.

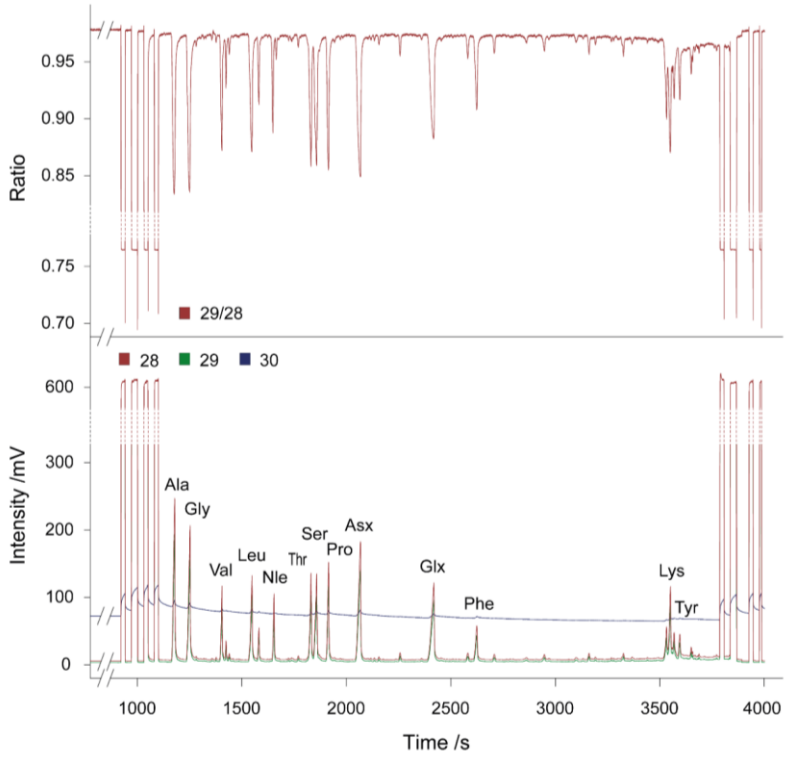


Figure 2.5. A characteristic GC-C-IRMS chromatogram depicting *N*-acetyl, *O*-isopropyl derivatised hydrolysable AAs in a soil sample. Also shown are the recorded ion current signals for each *m/z*, where the instrument is programmed to carry out N_2 analysis (*m/z* 28, 29 and 30). Above is the *m/z* 28 to 29 ratio for generating the $^{15}N : {}^{14}N$ isotope ratio. (Taken from Charteris *et al.*, 2016).

2.5 Data processing and calculations

2.5.1 AA quantification

$$\text{Apparent mass of sample AA} = \left(\frac{\text{sample AA peak area}}{\text{sample I. S. peak area}}\right) \times \text{mass of I. S.}$$

Equation 2.1

The quantification of AAs was achieved by comparing sample AA peak areas to that of the I.S., i.e. Nle, as shown by Equation 2.1. However, when different AAs are analysed at equal concentrations, structural variations between the AAs cause inequivalent responses on the GC-FID (Corr *et al.*, 2007a). To solve this, it is essential to calculate response factors (RFs) through the use of the AA standard mix of equal known concentrations (Eq. 2.2). The RFs can then be applied to determine the actual AA mass (Eq. 2.3), which are subsequently used to calculate the sample AA concentrations in mg of that AA per g of soil (Eq. 2.4).

$$\text{AA response factor} = \left(\frac{\text{standard AA peak area}}{\text{standard I. S. peak area}}\right)$$

Equation 2.2

$$\text{Actual mass of sample AA} = \text{apparent mass of sample AA} \times \text{AA RF}$$

Equation 2.3

$$\text{Concentration of sample AA} = \text{actual mass of sample AA} \times \frac{1000}{\text{total mass of hydrolysed sample}}$$

Equation 2.4

2.5.2 Isotopic calculations

The delta (δ) notation is conventionally used to report the isotopic abundances of substances. This differential notation was first officially defined by McKinney *et al.* (1950) to express the difference in isotope ratios, relative to a reference standard (Eq. 2.5a). Here, the δ value of isotope *A* of element *X* (e.g. $\delta^{15}N$) is calculated from the isotope ratio of the sample and the corresponding reference standard, R_{sample} and R_{std}, respectively. The internationally approved isotopic standard for N is AIR-N_2 (Mariotti, 1983); with a recommended value of 0.0036765 (Coplen *et al.*, 1992; Robinson, 2001; Meija *et al.*, 2016). The right side of Equation 2.5a is multiplied by 1000 and as such, values are expressed in parts per thousand – i.e. per mil (‰). As isotopic measurements obtained from GC-C-IRMS analyses are often expressed in terms of δ, the equation can be rearranged to calculate R_{sample} from its $\delta^{15}N$ value (Eq. 2.5b).

$$\delta^{A}X = \left(\frac{R_{sample}}{R_{std}} - 1\right) \times 1000$$

Equation 2.5a

$$R_{sample} = \left(\frac{\delta^{A}X}{1000} + 1\right) \times R_{std}$$

Equation 2.5b

Absolute abundances of isotopes are also commonly reported as atom fractions (or isotope-amount fraction). While the δ notation is practical for measuring and expressing small isotopic variations, with a precision that exceeds the uncertainty attained from measuring in atomic fractions, isotope δ values provide an inaccurate basis for tracer or amount-of-substance balance calculations (Brand and Coplen, 2012). This is because δ values are not linearly correlated to isotopic abundance, which is an atomic fraction. Thus, the use of atom fractions is preferable to δ values for more accuracy in tracer and isotope balance studies; measurements are made in δ values before conversion into atom fractions (Coplen, 2011). The atom fraction (AF) of ^{15}N relative to the total N in a sample is calculated using Equation 2.6.

$$AF = \frac{R_{sample}}{R_{sample} + 1}$$

Equation 2.6

Additionally, the atom fraction excess (AFE) is the difference in AF between a ^{15}N enriched soil, following incubation, and its equivalent control sample (Eq. 2.7).

$$AFE = AF_{(^{15}N)} - AF_{control}$$

Equation 2.7

2.5.3 N isotopic compositions and percentage fixations from bulk samples

The total number of moles of ^{15}N present per gram of bulk sample, $n(^{15}N)_{B.S.}$, is given by Equation 2.8 (Knowles, 2009). The percentage abundance of N, by mass, in the sample is denoted as % N and is obtained via elemental analysis of the bulk soil total N (TN) content.

$$n(^{15}N)_{B.S.} = AF_{(^{15}N)}\left(\frac{\%\ N}{1400}\right)$$

Equation 2.8

Subsequently, $n_E(^{15}N)_{B.S.}$ is the amount of ^{15}N present in excess moles per gram of a bulk ^{15}N-enriched sample, relative to the control samples (Eq. 2.9). This is equivalent to the number of moles of newly fixed ^{15}N present within the bulk enriched soil at that timepoint, i.e. how much $^{15}N_2$ has been fixed by active diazotrophs and measurable through the bulk sample.

$$n_{\mathrm{E}}(^{15}\mathrm{N})_{\mathrm{B.S.}} = \mathrm{AFE}\left(\frac{\%\ \mathrm{N}}{1400}\right)$$

Equation 2.9

The percentage of N_2 fixed in the bulk soil can also be calculated by following a series of equations. First, it is necessary to calculate the amount of ^{15}N applied to the sample. In this study, the ^{15}N-label is added in form of a gas; the ideal gas law (Eq. 2.10) can therefore be used to determine the number of moles of ^{15}N that was injected into the vial, i.e. $n(^{15}\mathrm{N})_{\mathrm{a}}$.

$$pV = nRT$$

Equation 2.10

$$n(^{15}\mathrm{N})_{\mathrm{a}} = \left(\frac{pV}{RT} \times (\%\ \text{of}\ ^{15}\mathrm{N}_2\ \text{in gas mix})\right) \times 2$$

Equation 2.11

As defined by the ideal gas equation, several factors are required in order to calculate $n(^{15}\mathrm{N})_{\mathrm{a}}$: p represents the total final pressure in the vial, measured after the ^{15}N-labelled gas had been injected; V is the total volume of the vial; R, the ideal gas constant; and T is the absolute temperature of the gas during the experimental procedure (taken as the laboratory temperature). The use of the ideal gas law, however, is also based upon the assumptions that: 1) atmospheric pressure is equal to 1 atm, and 2) the applied gas takes the form of an ideal gas. Finally, Equation 2.11 brings together the various parts, where the number of moles of applied $^{15}N_2$ gas is multiplied by a factor of 2 to account for the two atoms of N in each mole of $^{15}N_2$.

Finally, the percentage of N fixed in the bulk sample – taken as the % fixation of ^{15}N – may be calculated by using Equation 2.12.

$$\%\ \text{Fixation}\ ^{15}\text{N-B.S.} = \frac{n_{\mathrm{E}}(^{15}\mathrm{N})_{\mathrm{B.S.}}}{n_{\mathrm{E}}(^{15}\mathrm{N})_{\mathrm{a}}} \times 100$$

Equation 2.12

where $n_{\mathrm{E}}(^{15}\mathrm{N})_{\mathrm{a}}$ is the number of moles of excess ^{15}N applied to the sample (i.e. above substrate natural abundance values) (Eq. 2.13). This is calculated from: i) the number of moles of ^{15}N

applied per gram of dry soil, where $m_{\text{D.S.}}$ is the total mass of the soil sample when fully dried and grounded; and ii) the AFE of the applied $^{15}N_2$ gas, $\text{AFE}_{(^{15}N_2)}$. The latter value is obtained by subtracting the isotopic purity of the gas (i.e. $^{15}N_2$, 98 %) with the AIR-N_2 standard.

$$n_{\text{E}}(^{15}\text{N})_{\text{a}} = \frac{n(^{15}\text{N})_{\text{a}}}{m_{\text{D.S.}}} \times \text{AFE}_{(^{15}N_2)}$$

Equation 2.13

2.5.4 Percentage incorporations of the ^{15}N label into AAs

In compound-specific studies, the data may also be manipulated to show the percentage of the applied ^{15}N-isotope label incorporated into a certain soil AA – i.e. the % of ^{15}N fixed into each AA (Eq. 2.14; based on Knowles *et al.*, 2010):

$$\%\ \text{Fixation}\ ^{15}\text{N-AA} = \left(\frac{n(\text{N})_{\text{AA}} \times \text{AFE}_{(\text{AA})}}{n(^{15}\text{N})_{\text{a-H.S.}}}\right) 100$$

Equation 2.14

where the numerator in the fraction represents the ^{15}N enrichment of the AA. The total number of moles of N in the hydrolysed AA is presented above as $n(\text{N})_{\text{AA}}$ and its calculation may be simplified to result in Equation 2.15, where m_{M} is the molecular mass of that AA. AFE_{AA} indicates the AFE of the AA after incubation under $^{15}N_2$ compared with the control (Eq. 2.16). This equation is equivalent to Equation 2.7 and the AFs can be calculated from the AA's corresponding $\delta^{15}N$ value (Eq. 2.5b and 2.6).

$$n(\text{N})_{\text{AA}} = \frac{(\#\ \text{of N atoms in that AA})(\text{actual mass of sample AA})}{m_{\text{M}}}$$

Equation 2.15

$$\text{AFE}_{\text{AA}} = \text{AF}_{(^{15}\text{N-AA})} - \text{AF}_{\text{control-AA}}$$

Equation 2.16

Moving onto the denominator, $n(^{15}\mathrm{N})_{\text{a-H.S.}}$ is the number of moles of ^{15}N applied to the hydrolysed soil sample (Eq. 2.14). This value may be obtained using Equation 2.17, where $m_{\text{H.S.}}$ is the mass of the hydrolysed soil. Both $n(^{15}\mathrm{N})_{\mathrm{a}}$ and $\mathrm{AFE}_{(^{15}\mathrm{N}_2)}$ have been previously described in Equations 2.11 and 2.13, respectively.

$$n(^{15}\mathrm{N})_{\text{a-H.S.}} = n(^{15}\mathrm{N})_{\mathrm{a}} \times \mathrm{AFE}_{(^{15}\mathrm{N}_2)} \times \frac{m_{\text{H.S.}}}{m_{\text{D.S.}}}$$

Equation 2.17

Another way of presenting the data can be through calculating percentage retained ^{15}N incorporations (Eq. 2.18; Charteris, 2016). The percentage applied ^{15}N incorporation (Eq. 14) reflects how much ^{15}N has been fixed into the AA and the summation of these results for all hydrolysable AAs equates to the % of ^{15}N fixed into newly synthesised soil protein, based on the total amount of applied $^{15}\mathrm{N}_2$. However, if the applied ^{15}N has low bulk recovery or decreases over time, it is also possible to calculate % incorporations based on the number of moles of applied ^{15}N retained (above control or natural abundance soil values) in the system at that timepoint ($n_r(^{15}\mathrm{N})_{\text{a-H.S.}}$; Eq. 2.19). Percentage retention values will provide a different interpretation when compared to percentage fixation calculations as it is expressing how much of the total $^{15}\mathrm{N}_2$ fixed is actually incorporated into individual AAs and the soil protein pool.

$$\%\ \text{Retention}\ ^{15}\text{N-AA} = \left(\frac{n(\mathrm{N})_{\mathrm{AA}} \times \mathrm{AFE}_{(\mathrm{AA})}}{n_r(^{15}\mathrm{N})_{\text{a-H.S.}}}\right) 100$$

Equation 2.18

$$n_r(^{15}\mathrm{N})_{\text{a-H.S.}} = n_{\mathrm{E}}(^{15}\mathrm{N})_{\mathrm{B}} \times m_{\text{H.S.}}$$

Equation 2.19

3 Initial method development using clover root nodules and peat

Symbiotic N-fixers such as clover and other legume plant families have been studied closely for many decades. Ever since rhizobia was recognized as the main source of fixed N in legumes, root nodule development and the rhizobia-legume interaction have become key to BNF research (Hirsch *et al.*, 2001). In the development of a new ^{15}N-SIP method for the quantification of BNF in soils, symbiotic N-fixers were therefore a good starting point as information is more widely available in literature (Hardy *et al.*, 1968; Peoples *et al.*, 1989; Ledgard and Steele, 1992; Kahindi *et al.*, 1997; Kiers *et al.*, 2003; Rejili *et al.*, 2012). Clover plants were chosen due to their accessibility; previous ARA studies on the species provided further helpful guidelines for the method development (Allison *et al.*, 1942; Dart and Day, 1971; Francis and Alexander, 1972; Huss-Danell *et al.*, 2007). Ensuing results could also initiate direct comparisons of the different techniques for quantifying N_2 fixation.

Similarly, it was important to verify the viability of the pilot compound-specific ^{15}N-SIP method on soil from other types and settings as well. Free-living diazotrophs represent a large pool of N-fixers, so an effective method for measuring N_2 fixation would need to capture the activity of all active diazotrophs, regardless of their habitat or ecosystems. Peat was chosen for this initial experiment because of its acidity and organic-rich composition. An assessment of indicator genes in soils sampled from various landscapes across Britain by Malik *et al.* (2017) revealed that low-pH soils, typically also comprising higher moisture and organic matter content, possessed a larger abundance of N_2 fixation genes; thus, indicating that such soils may demonstrate greater N-fixing activity, whether through symbiotic or non-symbiotic paths. Results obtained from this experiment would then allow for further method refinement and a deeper understanding of free-living N-fixers.

3.1 Considerations for method development

For closed-system soil incubations, an important factor to consider is the process of soil respiration and its effects on soil microbes. As a key ecosystem process, soil respiration releases C in the form of CO_2 as a by-product of below-ground metabolisms in the soil and litter layers. A problem arises during incubations in closed vessels – especially incubations over longer time periods – as the CO_2 pressure can accumulate and alter ambient soil conditions, such as changes

in pH (Luo and Zhou, 2006). It was also important to establish an equilibrium between the requirement of O_2 for microbial metabolism and the O_2-sensitive trait of the nitrogenase enzyme. Consequently, in order to prevent the build-up of CO_2 inside the vial and limiting the microbial system from going anaerobic too quickly, the produced CO_2 had to be captured or removed from within the sealed vials. A CO_2 trap was therefore created, containing ground NaOH as the absorbent. The theory behind this technique is similar to those employed in studies on carbon capture and storage as a way of reducing CO_2 emissions from fossil fuel consumption (Sanz-Pérez *et al.*, 2016). While many techniques have evolved to remove the CO_2, researchers have focused on the use of NaOH due to its rapid reactivity and cost-effectiveness (Stolaroff *et al.*, 2008; Yoo *et al.*, 2013; Shim *et al.*, 2016). In this reaction, gaseous CO_2 is converted to sodium carbonate (Na_2CO_3), and ultimately sodium bicarbonate ($NaHCO_3$), by the NaOH absorbent (Eq. 3.1 to 3.3). Anaerobic indicator strips were also employed throughout the duration of the incubations to monitor O_2 depletion and observe the state of the system throughout the incubation period.

$$CO_2 + 2NaOH \rightarrow Na_2CO_3 + H_2O$$

Equation 3.1. Initial chemical reaction of CO_2 absorption reaction by NaOH.

$$Na_2CO_3 + CO_2 + H_2O \rightarrow 2NaHCO_3$$

Equation 3.2. Second range reaction of overall absorption reaction as CO_2 is continuously absorbed.

$$CO_2 + NaOH \rightarrow NaHCO_3$$

Equation 3.3. Overall CO_2 absorption net reaction, when NaOH becomes the limiting reactant.

3.1.2 Testing the viability of the proposed CO_2 traps

Incubation vials were filled with soil (*ca.* 7 g; see Section 3.2.1 below for soil description) and then tightly stoppered and sealed. CO_2 traps were inserted singly into half of these vials, before leaving all the vials to sit at room temperature. A Hamilton samplelock™ syringe (100 µL) was then used to collect a small amount of the vial headspace at the designated timepoints, starting from $t = 0$. The sample headspace was first pressurized in the syringe, before briefly opening the valve in order to release excess pressure and equilibrate the sample with atmospheric pressure. Shortly after, the sample was then swiftly injected through a GC injection port containing a butyl

rubber septa seal, at 125 °C. Samples were also taken from control vials (i.e. vials without a CO_2 trap) for valid comparisons.

3.1.2.1 Measuring CO_2 gas concentrations

The CO_2 gas concentrations were measured using a Carlo Erba HRGC5300 gas chromatograph, equipped with a Porapak® QS packed column (3 mm x 4 m). A N_2 carrier gas was used, with a flow rate of 35 mL min^{-1}. The GC was fitted with both a nickel oxide (NiO_2) catalyst methaniser and a flame ionization detector (FID). Support gases for the FID included H_2 at 30 mL min^{-1}, and zero air at 400 mL min^{-1}. Gas concentrations were calibrated against speciality gas standards of 500 ppm and 10,000 ppm CO_2 (BOC, Guldford, UK), with a relative analytical precision (coefficient of variation, CV) of usually <2 % based on multiple injections.

3.2 Sample collection and preparation

3.2.1 Clovers

Clover plants were sampled randomly in early April 2018 from a trial plot of the Rothamsted CINAg project, near the North Wyke Farm Platform in Okehampton, Devon, UK. The plants are part of the AberClaret red clover variety and are grown in pelo-stagnogley soil (a clayey non-calcareous soil) of the Hallsworth series (British Classification). At the time of sampling, the clover root nodules appeared to be on the smaller side and were not yet well-developed. Roots which did contain nodules were gently disentangled and excised from the plant, before being carefully rinsed first in tap water, then DDW, and gently patted dry with a paper towel. Each incubation vial contained 200 mg of clover roots – with approximately the same number of nodules in each vial – mixed with 7.5 g of soil taken from the same site.

3.2.2 Peat

Fieldwork was completed in mid-June 2018 to collect peat soil in Exmoor National Park, located in the southwest of England (51° 7'10.21"N, 3°45'2.18"W). The sampling site was near Simonsbath in central Exmoor (the sampling location is also known as Spooner's catchment). The site was also ideal in being one of the few accessible areas of natural peatland with a relatively

small fraction of undisrupted peat (i.e. by drainage or other anthropogenic factors). An assortment of natural bog flora, comprising of the *Sphagnum* and *Eriophorum* species, remained prevalent throughout this section. A more detailed site description can be found in the work conducted by Tian (2014; Site 7).

Two locations were sampled randomly within a few meters of each other along the catchment transect. Peat soil within the catchment is acidic, providing an average pH of 4.0. Sample peat monoliths of 0–30 cm depth was taken from each location using a shovel, before being roughly sawed into two separate sections of surface peat (0–10 cm) and deeper peat (10–30 cm). This initial experiment focused on examining surface peat based on studies by Warren *et al.* (2017); ARA and *nifH* gene transcription had detected diazotroph activity solely in these samples. Surface peat samples were homogenised as gently and non-destructively as possible to avoid rupturing the *Sphagnum* tissues and disturbing the microbial community. Bulk peat (5 g) were subsequently placed into 30 mL glass serum vials in preparation for the incubations.

3.3 Experimental methods

Unless stated otherwise, a full description of the incubation experiments, AA protocol and instrumental analyses have been described in Chapter 2.

3.3.1 Incubations and sample preparation for analyses

3.3.1.1 Clover root nodules

Incubation experiments were performed in triplicate (i.e. three sample vials for each timepoint) and were terminated by immersion in liquid N_2 after time periods of 1, 2, 4, 8 and 24 h. A set of samples at time $t = 0$ were used as controls.

3.3.1.2 Peat

The peat incubations were carried out over a longer duration – being halted only after 1, 2, 4 and 8 days. Peat soils at natural abundance levels (i.e. $t = 0$) were also analysed as controls. Further

information on the subsequent sample preparation, extraction and derivatization procedures were as described in Sections 2.2 and 2.3.

3.3.2 Instrumental analyses

Details of bulk soil $\delta^{15}N$ analyses, GC-FID and GC-C-IRMS analyses are outlined in Section 2.4, alongside relevant calculations in Section 2.5.

3.4 Results

3.4.1 Capturing CO_2 with NaOH

Results from the tests shows that the developed CO_2 trap was effective in removing CO_2 from the closed incubation system. Without the trap, the concentration of CO_2 inside the sealed vial increased linearly with time (Fig. 3.1a). On the other hand, the vial which had an attached CO_2 trap revealed a decrease in CO_2 concentrations overtime – until reaching a plateau after about 5 h (Fig. 3.1b). This illustrates that the use of CO_2 traps can therefore delay the build-up of gaseous CO_2 within the incubation vials and prevent the system from instantly going anaerobic.

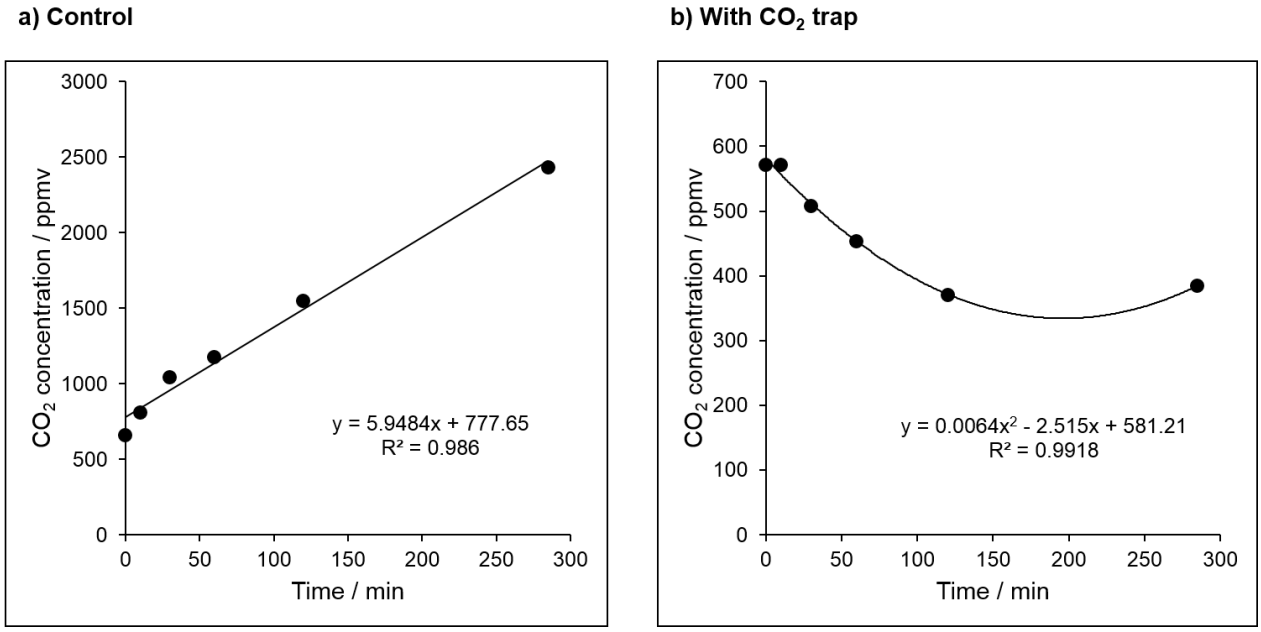


Figure 3.1. CO_2 concentrations inside sealed vials with a) no CO_2 trap, and b) a CO_2 removal trap, over time.

3.4.2 Clover root nodules

Bulk ^{15}N analysis on clover roots and its nodules showed an increase in $\delta^{15}N$ enrichment over time (Fig. 3.2). The mean $\delta^{15}N$ value at $t = 0$ refers to natural abundance levels, while the elevated $\delta^{15}N$ values at subsequent timepoints verify the presence of $^{15}N_2$ gas being fixed by the clover root nodules. There is a sharp increase from $t = 0$ to $t = 1$, going from 0.92 ‰ to 5.21 ‰. This was followed by a more gradual increase to reach a peak of 7.52 ‰ at $t = 8$, before development of a plateau. Comparison with the ARA carried out by Turpin-Jelfs (2017; Figure 3.3) exposes a consistent trend in the N_2 fixation activity of clover root nodules. Both experiments followed similar incubation conditions – though one noticeable difference would be that for ARA, the amount of C_2H_4 produced occurred in rather evenly paced increments throughout the timepoints; whereas results from bulk ^{15}N analysis displayed fast incorporation over the first 4 h, then became more stable for the remainder of the experiment.

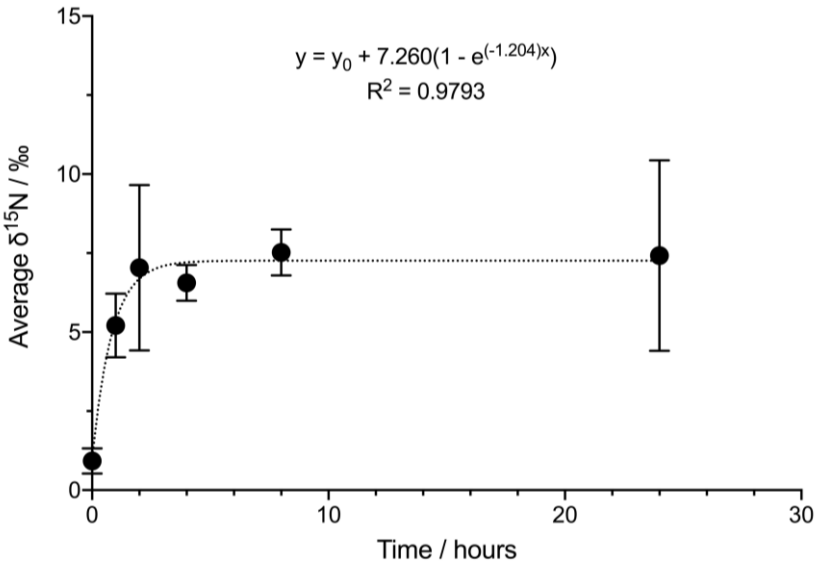


Figure 3.2. Mean bulk $\delta^{15}N$ values for clover roots and its nodules, over a 24 h incubation experiment. Error bars represent the standard error of the mean (SEM) of triplicate incubations of each individual timepoint (i.e. n = 3). The non-linear regression displayed is a single first-order exponential assimilation of the form: $\Delta^{15}N = \Delta^{15}N_0 + P(1 - e^{-k_1 t})$, where $\Delta^{15}N_0$ is the rate of ^{15}N incorporation at 'steady state' (i.e. by definition $\Delta^{15}N_0 = 0$), P is the plateau level reached and $k1$ is the 1st-order rate constant (Charteris, 2016).

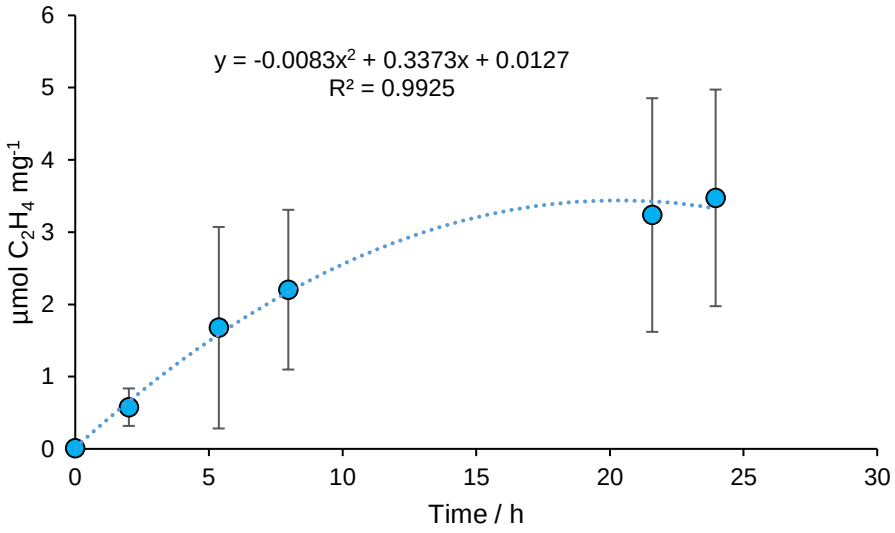


Figure 3.3. Mean C_2H_4 produced over a 24 h incubation period, where error bars represent mean absolute deviation (Turpin-Jelfs, 2017, unpublished data).

Typical gas chromatograms for the prepared AA standards and the clover root nodules are shown in Figure 3.4 and 3.5, respectively. The chromatograms of the clover plants in study were able to match well with the established elution order of the AA standards. Masses of each individual hydrolysable AA in the clover roots and nodules were quantified using the known amount of Nle I.S. added at the start of the extraction (see Section 2.5.1 for calculations). Figure 3.6 displays the average concentrations of each hydrolysable AA after incubation for 24 h. Compared to other AAs, Asx showed the highest concentration, with Glx coming in second. This also consistent with literature where AA profiles obtained from white clover (*Trifolium repens* L.) by Lesuffleur and Cliquet (2010) revealed asparagine (Asn) as the major AA in both root tissue and nodules, followed by glutamate (Glu), aspartate (Asp) and glutamine (Gln). In our study, a comparable pattern is observed in the higher concentration of Asx, which is a combination of Asp and Asn as a result of acid hydrolysis; similarly, combination of Glu and Gln yields the large proportion of Glx seen in Figure 3.6.

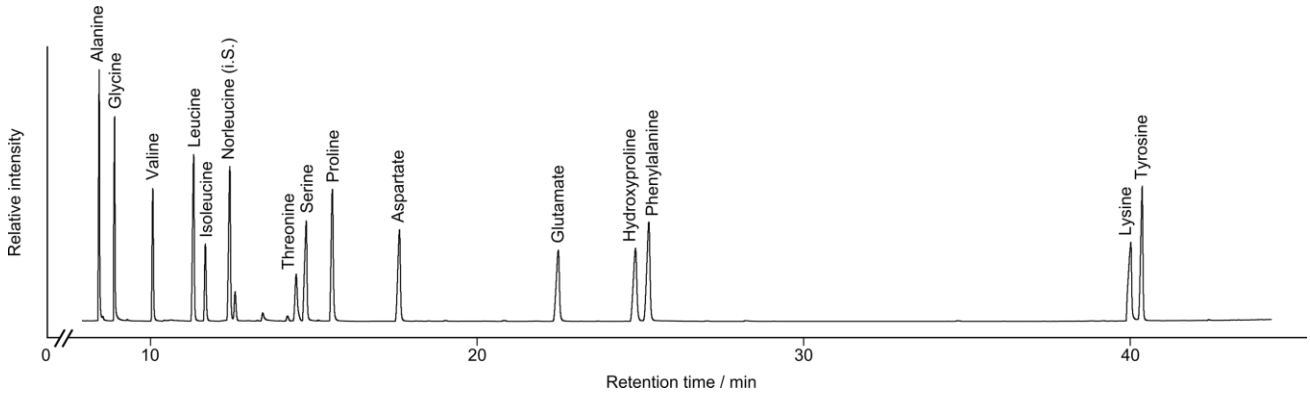


Figure 3.4. An exemplar gas chromatogram of the extracted AA standards, showing their order of elution.

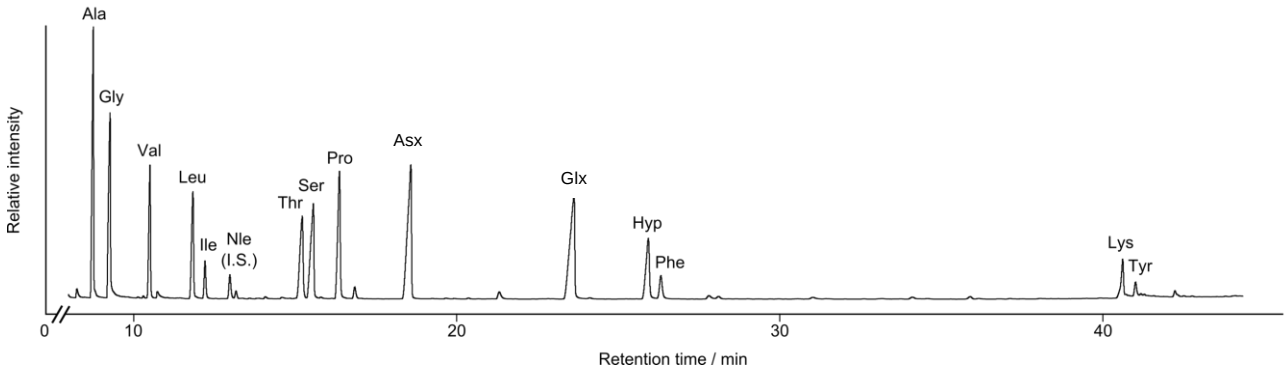


Figure 3.5. Characteristic gas chromatogram for hydrolysable AAs found in clover roots and their nodules.

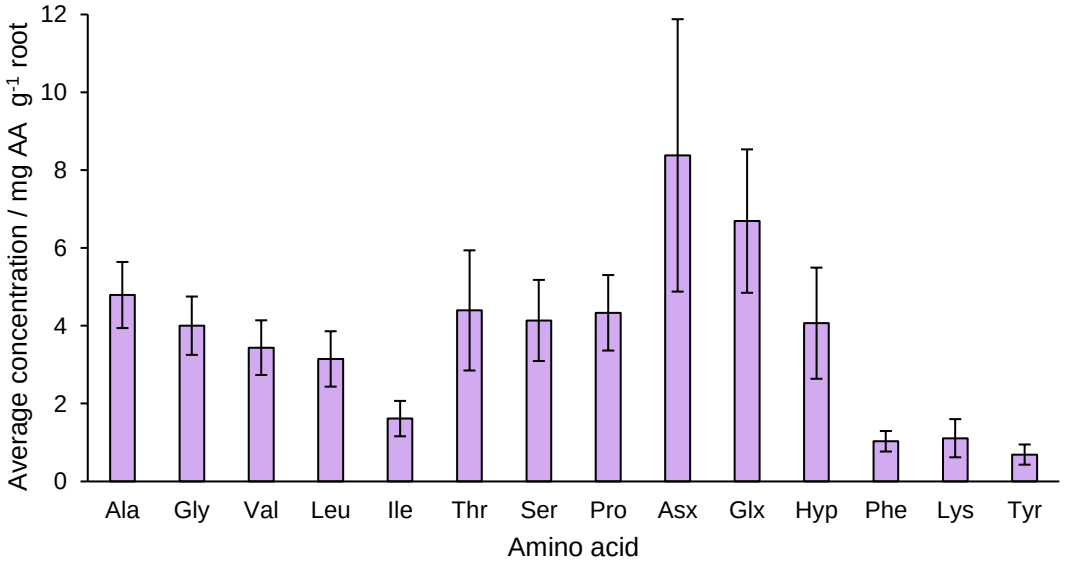


Figure 3.6. Average concentration of individual AAs in clover roots and its nodules, after a 24 h incubation. Concentrations are expressed in mg of that AA per g of the sample. Error bars are ± SEM (n=3).

Average $\delta^{15}N$ values from the compound-specific ^{15}N-SIP approach obtained using GC-C-IRMS also presented an overall increase in enrichment (Fig. 3.7). Glx displayed the highest increase; in the first few hours, assimilation into Glx occurred more rapidly than into any other AA. Incorporation of the ^{15}N-tracer into each AA was evidently faster at the start of the incubations. There was little difference between average $\delta^{15}N$ values at $t = 8$ and $t = 24$ – suggesting that the system had plateaued after just 8 h into the experiment. This observation is also consistent with results from the bulk ^{15}N analyses, which showed a comparable trend (Fig. 3.2).

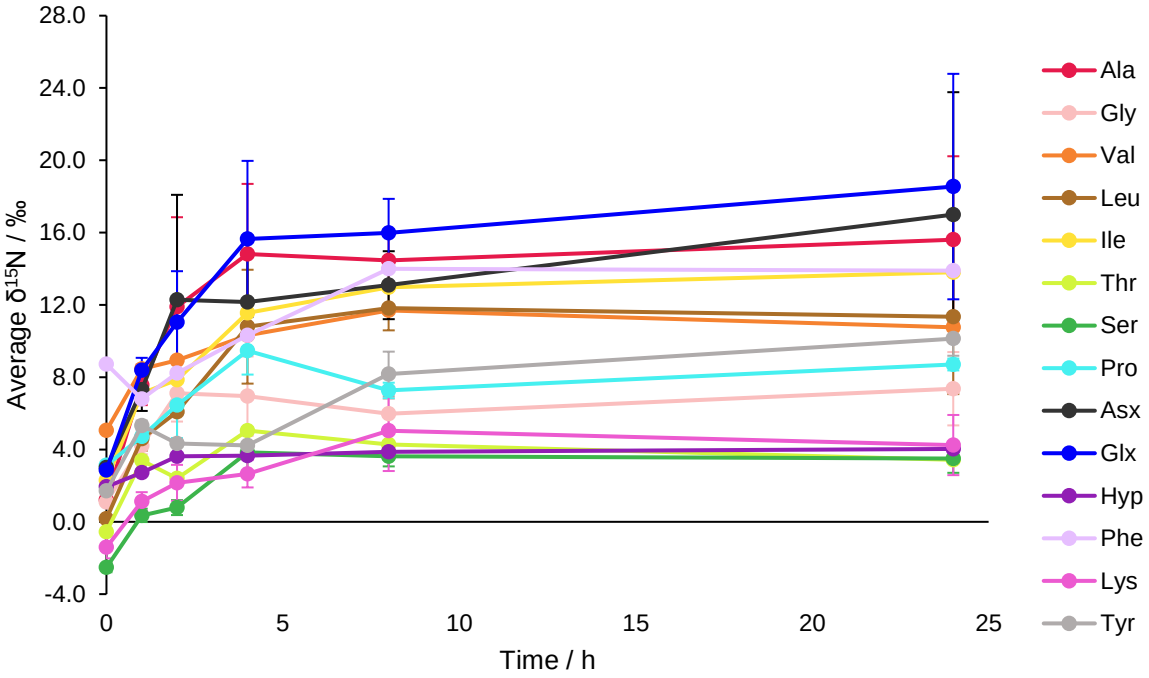


Figure 3.7. $\delta^{15}N$ values of individual AAs in clover roots and its nodules over the course of a 24-hour incubation experiment. Error bars are ± SEM (n=3).

3.4.3 Peat

Fifteen AAs were identified in surface peat soil of 0–10 cm in depth from both sites. An exemplar gas chromatogram for surface peat soil (0–10 cm depth) is shown in Figure 3.8. The peaks are similar to the pattern produced by the clover root nodules, except for Hyp. Overall, the proportion of acidic AAs (i.e. Asx and Glx) and neutral AAs were most abundant, while Hyp and the two aromatic AAs – Phe and Tyr – were found in smaller proportions (Fig. 3.9). This trend also corresponds to GC-MS analyses performed by Kunnas and Eronen (1994) on a Finnish peat bog at various depths. The study found that surface peat consisting of *Sphagnum* moss contained more acidic AAs than neutral AAs; the amount of acidic AAs was revealed to decrease when moving down the soil profile and as the system became anaerobic.

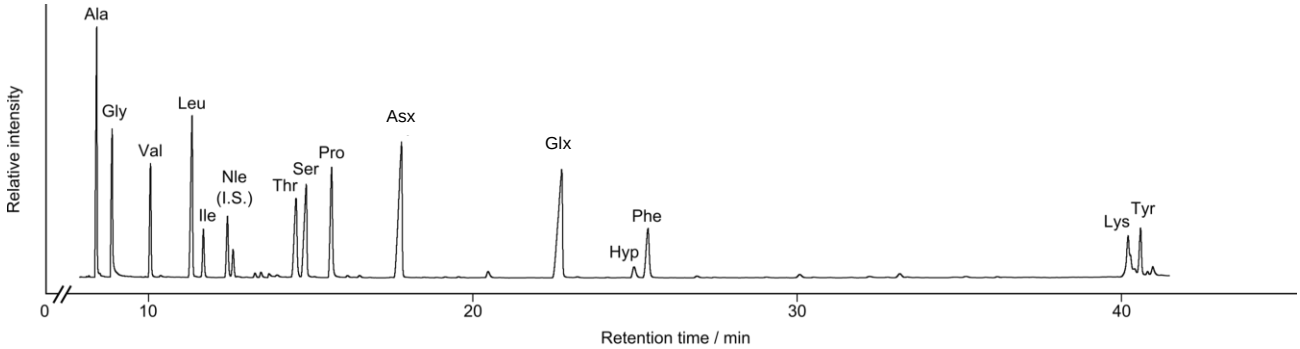


Figure 3.8. Characteristic gas chromatogram for AAs found in peat soil.

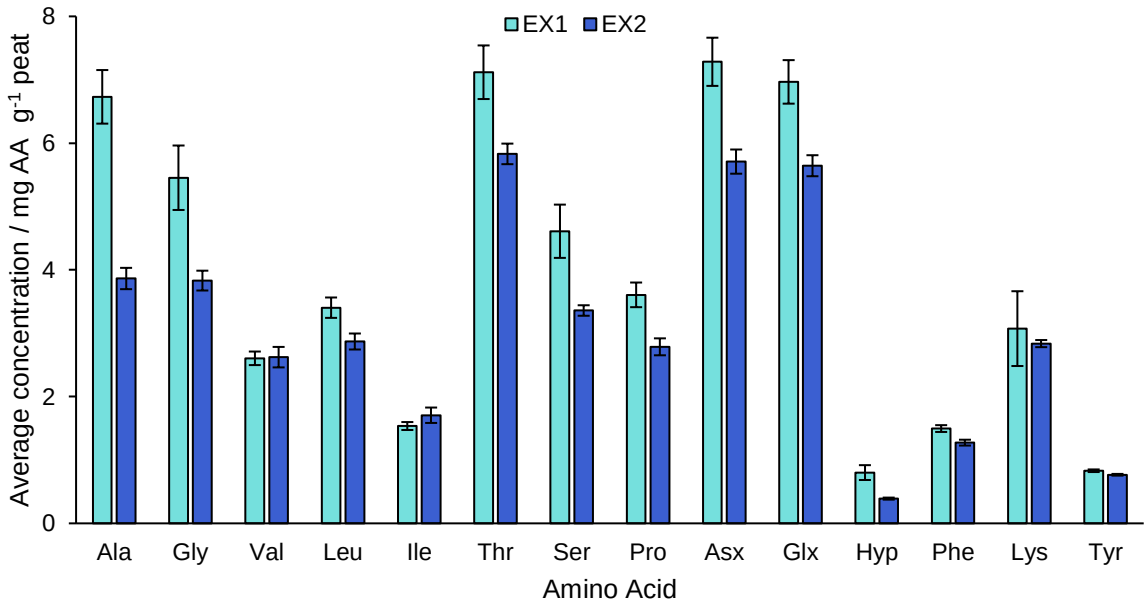
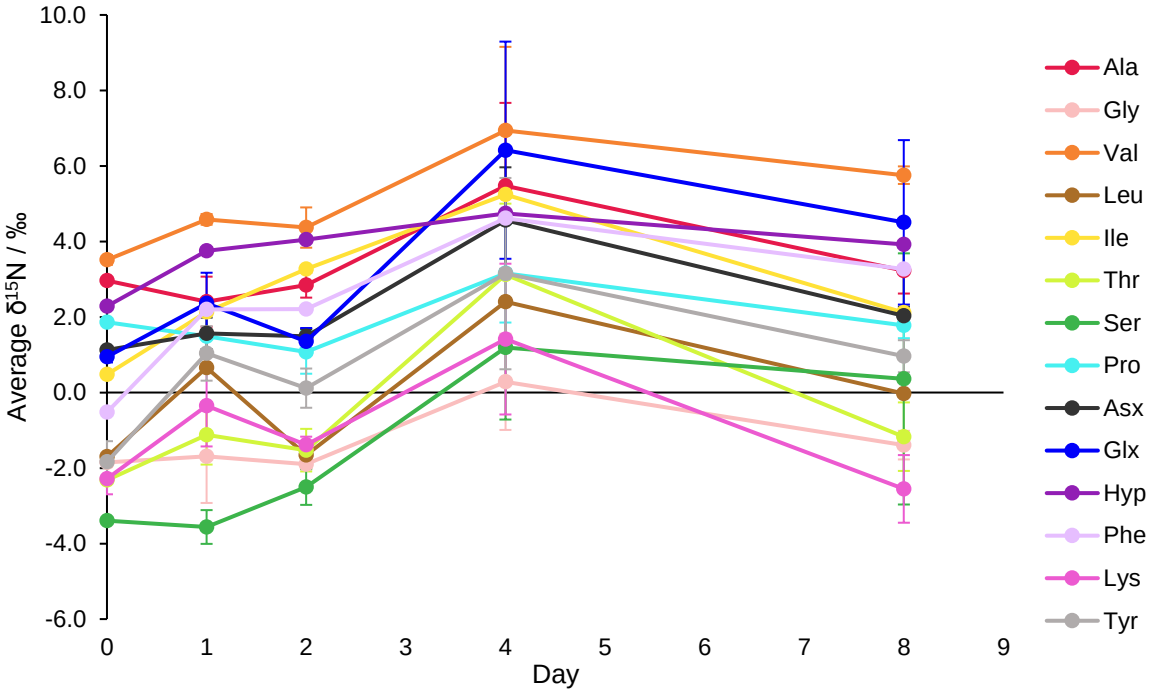


Figure 3.9. Average concentration of individual AAs in peat soil, after 8 days. Error bars are ± SEM (n=3).

The compound-specific ^{15}N-SIP approach using GC-C-IRMS produced mean δ^{15}N values for all the major proteinaceous AAs; however, Figure 3.10 shows only slight shifts in δ^{15}N enrichment values between control and incubated peat soils from both sites. For Site EX1, values showed highest levels of enrichment on day 4, followed by a drop to natural abundance levels on day 8 (Fig. 3.10a). On the other hand, δ^{15}N values for Site EX2 were even lower – showing little to no increase throughout the entirety of the experiment (Fig. 3.10b). Thus, preliminary δ^{15}N data appears to oppose the initial premise that peat soils have high N_2 fixation activity as reflected in the large abundance of *nif* functional genes. While Site EX1 depicts slightly more variations between each day, δ^{15}N values for Site EX2 remain rather constant throughout the whole experiment. Initial deductions from these results would therefore suggest low nitrogenase activity in the sampled peat as determined by the developed compound-specific ^{15}N-SIP method.

a) Site EX1



b) Site EX2

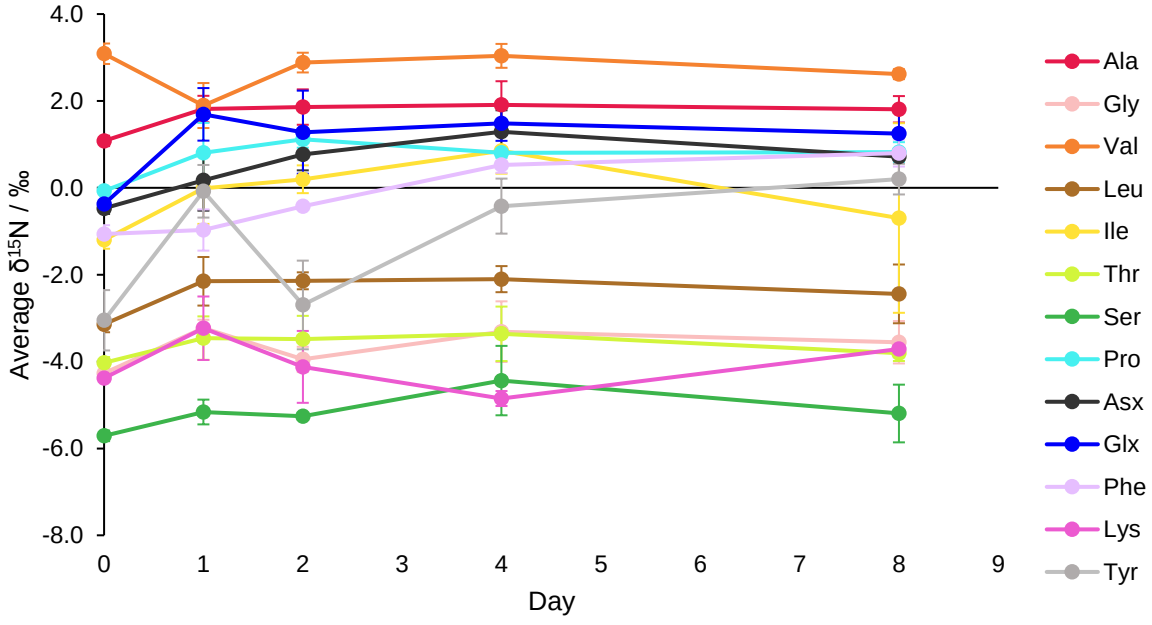


Figure 3.10. $\delta^{15}N$ values of individual AAs over the course of an 8-day incubation experiment for surface peat soil from Sites EX1 and EX2. Due to its low concentration, hydroxyproline (Hyp) could not be accurately quantified and is therefore excluded from Site EX2. Error bars are ± SEM (n=3).

3.5 Discussion

3.5.1 Clover root nodules

3.5.1.1 Hydroxyproline in legume root nodules

Hydroxyproline (Hyp) is known to be present in very low abundances in plants, although GC-FID analyses of the clover root nodules showed contrasting results. This particular AA is generally found in collagen located in bones, muscles, ligaments, and tendons – accounting for approximately 11 % of AAs (Kendall *et al.*, 2017). Interestingly, results from this experiment showed that Hyp appeared as a significant component in all the chromatograms of clover roots and its nodules (Fig. 3.5) and its concentration after 24 h was also rather high (Fig. 3.6).

Several studies have shown that legume root nodules, which offer a niche for symbiotic N-fixing rhizobia, are rich in Hyp. In plants, Hyp-rich glycoproteins (HGRP) can be ascribed to extensins of the plant cell wall and arabinogalactan proteins (AGPs), which have important roles in several aspects of plant development and growth (MacLean *et al.*, 2009). Analysis of leguminous plants nodules have also detected Hyp (Cassab, 1986; Nap and Bisseling, 1990; Frueauf *et al.*, 2000; Rathbun *et al.*, 2002). Frueauf *et al.* (2000) reported Hyp-rich extensin as the major structural protein in nodules and root cell walls of *Medicago truncatula*, a leguminous plant, while Cassab *et al.* (1985) noticed that the concentration of Hyp increased considerably during the development of the soybean root nodules. Taken together, the finding high abundances of Hyp present in the AA extracts of clover root nodules is not surprising.

3.5.1.2 Compound-specific ^{15}N-SIP vs. bulk ^{15}N analyses

EA-IRMS operates by combusting the entire sample, as opposed to a single component; hence, all compound-specificities are lost with bulk analyses. Although the elevated δ^{15}N values provided by bulk analysis do verify the presence of the added ^{15}N tracer fixed by the root nodules, no information regarding the form or internal processing of the fixed ^{15}N may be gained. On the other hand, compound-specific ^{15}N-SIP offered preliminary insights into individual AAs through following their enrichment. In Figure 3.6, the elevated concentrations of Asx and Glx after 24 h reveal their key presence in clover roots and its nodules. In Figure 3.7, assimilation into Glx happened most rapidly, in comparison to the other AAs. While this experiment is still

insufficient to arrive at definite conclusions, the results do suggest that these two AAs may play a leading role in initial microbial assimilation processes taking place after the $^{15}N_2$ had been fixed. Both Glu and Asp are also recorded in literature as some of the early AA products of N_2 fixation in legume root nodules (Grimes and Fottrell, 1966). Thus, the possibility to reveal such linkages represent a major advantage of compound-specific isotope analyses.

Compound-specific ^{15}N-SIP using GC-C-IRMS not only reveals pathways of isotope routing, but also achieved higher δ^{15}N enrichment values overall. For example, in comparing the δ^{15}N values at $t = 24$ of both instrumental methods, bulk N isotope analysis attained a value of 7.4 ‰ (Figure 3.2), whereas GC-C-IRMS analyses produced results of over double that value for most AAs (Figure 3.7). Bulk analyses therefore have lower precision and discriminative ability, especially in cases where limited ^{15}N-labelling levels are attained. This emphasises the value of compound-specific ^{15}N-SIP using GC-C-IRMS in achieving greater sensitivity for tracing ^{15}N substrates applied at lower enrichments, further underlining its utility as a tool for measuring N_2 fixation.

Nevertheless, the relatively low levels of enrichment detected in the clover roots and its nodules raises questions regarding the viability of the developed method. Symbiotic diazotrophs have been estimated to contribute to roughly 80 % of biologically-fixed N in agriculture (O'Hara, 1998). Knowing this, one could expect higher levels of ^{15}N-enrichment in these incubation experiments. The amount of BNF achieved by symbiotic N-fixers can, however, vary greatly depending on an array of factors including legume persistence, environmental conditions and soil properties (Ledgard and Steele, 1992). As the pilot experiment took place over merely 24 h, there may not have been sufficient time for the microbial system within each incubation vial to respond and equilibrate to their new conditions. It is also possible that some root nodules in the experiment were inactive or contained ineffective strains of rhizobium. Overall, the physiological properties of N_2 fixation have to be further explored in order to formulate a quantification method that will effectively quantify this biological process. Yet, the visible rise in δ^{15}N values for both bulk and compound-specific ^{15}N-SIP analyses can be considered as positive signs that the method development is progressing in the right direction.

3.5.2 Peat

3.5.2.1 Biological nitrogen fixation in peatlands

There are several possible explanations regarding the low N_2-fixing activity witnessed in this pilot experiment (Fig. 3.10). It may be important to bear in mind that peatland ecosystems operate on a much slower timescale. While organic matter is cycled relatively quickly in most tropical and temperate ecosystems, C and N are stored in peatlands for millennia (Fowler *et al.*, 2013). Thus, the time taken for fixed N to be properly assimilated into the decomposing *Sphagnum* litter and lower peat layers may not be a fast exchange, but rather a process that occurs over a time-span of several days, at the very least (Larmola *et al.*, 2014). In the process of method development, it is therefore necessary not only to take account of the physiological properties of N_2 fixation, but to understand the particular soil system being studied as well.

The extremely low enrichment of $\delta^{15}N$ values observed in this initial experiment contrasts with reports on the abundance of free-living N-fixers generally present in peatlands. Various genetic studies on peatlands-associated microbial communities have revealed a high diversity of N-fixing diazotrophs (Bragina *et al.*, 2012; Vile *et al.*, 2014; Warren *et al.*, 2017). However, the rich presence of nitrogenase-encoding *nif* genes may not always equate to higher N_2 fixation activity. A study by Mäkipää *et al.* (2018) on decaying Norwegian spruce logs revealed that despite the higher abundance of *nifH* copies at later phases of decay, no correlation was found between the copy numbers and the amount of N being fixed. While this is not a direct example, the recovered *nifH* sequences from decomposing wood all belonged to *Alphaproteobacteria (Rhizobiales)* – which is also the dominant class of N-fixing bacteria found in peatlands (Warren *et al.*, 2017). However, this serves as just one explanation for the low levels of enrichment from the peat incubations; the main concern lies in the appropriateness of the initial method used.

4 Further method refinements: A closer look into the physiology demands of nitrogenase

The physiology and functional biology of diazotrophs are significantly affected by the properties and needs of the nitrogenase enzyme. Therefore, to better understand N_2 fixation – and develop an effective quantification method for the process – we must take a closer look into nitrogenase. This chapter examines the physiology of this enzyme in close detail, by focusing especially on the functional controls for free-living N-fixers. The findings are then selectively applied to create an improved method that is once again tested on surface peat.

4.1 Functional controls for free-living nitrogen fixers

Despite their diversity as a group, the productivity of all non-symbiotic N-fixers are highly dependent on the properties and requirements of the nitrogenase complex. Physiological studies have been conducted in attempt to elucidate the responses of diazotrophs to transformations in their environment by exploring their genetic, structural and biochemical make-up. Thus, much focus has been placed on nitrogenase as the catalytic enzyme facilitating BNF. The enzyme's activity is primarily characterised by: i) exceptional sensitivity to O_2, ii) the metal content of the enzyme's component proteins, iii) the need to have sufficient supplies of ATP and reducing power to support N_2 fixation, and lastly, iv) common suppression by the availability of N (Hill, 1992; Reed *et al.*, 2011).

4.1.1 Oxygen

The level of O_2 present in the biological system has a significant impact on the efficiency with which diazotrophs fix N_2 (Stewart, 1969). Free-living N-fixers live in a variety of environments which extend over differing gradients of O_2 availability (Reed *et al.*, 2011). Nevertheless, O_2 has the ability to inhibit the nitrogenase enzyme and hence suppress N_2 fixation – so particular diazotrophs only fix N_2 under strictly anaerobic conditions (Rubio and Ludden, 2008). Some N-fixers evade the potentially toxic effects of O_2 via techniques ranging from the time separation of N_2 fixation from O_2-evolving processes like photosynthesis, through to the intensification of respiration to diminish O_2 levels (Robson and Postgate, 1980). Obligate aerobes, however, establish optimal conditions for diazotrophy where O_2 supplies are leveled with the respiratory

demand. At elevated concentrations of O_2, inhibition may occur through the reduction of both nitrogenase production and nitrogenase activity (Hill, 1992). The O_2-sensitive trait of nitrogenase therefore imposes substantial physiological constraints on N_2 fixation as there is a need to shield the enzyme from O_2-damage (Dixon and Kahn, 2004). With this in mind, it may be more fitting to carry out longer incubations (e.g. over two weeks) to allow for the system to draw down its O_2 level and effectively become anaerobic.

4.1.2 Metal content of component proteins

Metal bioavailability presents another limitation to N_2 fixation in both natural and managed ecosystems. All three of the currently identified forms of nitrogenase require Fe, while the rest also involve Mo or V (Bellenger *et al.*, 2011). A range of genetic and environmental factors – such as the availability of the metal, light and O_2 concentrations – control which type of nitrogenase is biosynthesised. Nevertheless, it is commonly accepted that N_2 fixation occurs most efficiently with the Mo-nitrogenase; when Mo is scarce, N-fixers then express alternative nitrogenases containing V or Fe (Masepohl *et al.*, 2002; Bellenger *et al.*, 2011). Yet, as the least abundant trace metal in soils, Mo could potentially be the limiting element for diazotrophic activity (Wichard *et al.*, 2009).

The low pH conditions present in peatlands would be expected to favour N_2 fixation by alternative nitrogenases, where [Fe] > [V] > [Mo] (Warren *et al.*, 2017). However, in their study of diazotrophy in a *Sphagnum* peatland, Warren *et al.* (2017) proved the opposite – nutrient profiles and nitrogenase expression showed that peatland diazotrophs actually favoured the Mo-based form of the enzyme. The question of how these N-fixing bacteria gain access to rare supplies of Mo remains uncertain; though it has been suggested that Mo can be retained by binding to SOM, to later be captured by diazotrophs (Wichard *et al.*, 2009; Marks *et al.*, 2015). Thus, it is possible that the availability of relevant trace elements may not be a major limitation for N_2 fixation by peat soils.

4.1.3 ATP

N_2 fixation is one of the most energetically demanding processes in nature. The strong triple bond makes N_2 an extremely stable compound and its reduction is therefore accompanied by a

high activation energy. At least 16 mol of ATP is required to fix one mol of N_2 (Fisher and Newton, 2002); this translates to expending 100 grams of glucose per gram of N_2 fixed (Reed *et al.*, 2011). The reaction hydrolyses a minimum stoichiometry of two ATP for every electron transfer during each step of the nitrogenase catalytic cycle (see Eq. 1.1). These high-energy demands are met by N-fixers through various means, whether by utilising energy supplied via photosynthesis or derived from the metabolism of SOM (DeLuca *et al.*, 1996). Consequently, as the reaction is so kinetically demanding, the efficiency of N_2 fixation is largely dependent on availability of this reducing power.

The addition of an external energy source is known to help drive N-fixing activity. In studies conducted by Kravchenko and Doroshenko (2003), it was initially reported that the incorporation of $^{15}N_2$ tracer gas into the upper layers of peat soils produced low total amounts of fixed N by the end of a 15-day incubation period. However, the addition of glucose successfully stimulated N_2 fixation and led to increased nitrogenase activity over the same time period. A second addition of the sugar proved more effective by immediately driving N_2 fixation levels even higher (Fig 4.1). All the more, this shows that not only is N_2 fixation catalysed by energy, but the whole process practically fails to occur without it.

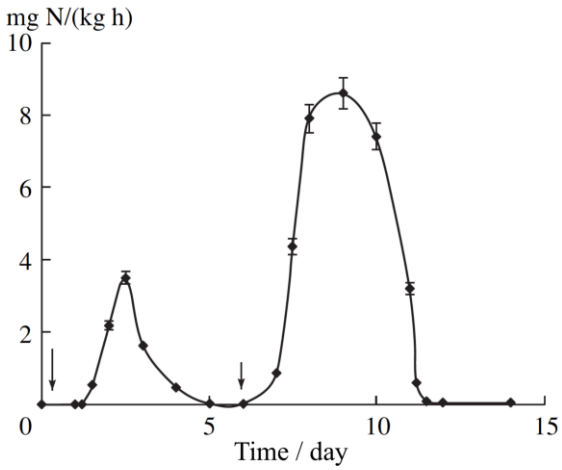


Figure 4.1. A time-course showing the nitrogen-fixing activity of peat soil (10–20 cm depth) followed using the acetylene method. Arrows signify the moments where glucose is added to the peat samples. (Taken from Kravchenko and Doroshenko, 2003).

In this chapter, the hypothesis is tested that introducing an energy supply to the incubations will increase N_2 fixation, to produce an effective assay.

4.2 Experimental methods: the second edition

Now equipped with a deeper understanding of the properties and requirements of the nitrogenase enzyme, these findings can be applied in the development of a more effective quantification method for BNF. It appears that the main factors to consider include: i) the level of O_2 present in the microbial system, and ii) the amount of energy available for the reaction to effectively take place. Based on these conditions, major refinements to the method will be to ensure anaerobicity by implementing longer incubation times and provide an external source of energy for the soil microbial system in order to boost N_2-fixing activity.

4.2.1 Preparation of the energy source

Glucose (Glc; Fisher Scientific, Loughborough, UK) was dissolved in DDW to provide a solution with a final concentration of 10 mg C mL^{-1}, based upon the procedure carried out by Kravchenko and Doroshenko (2003).

4.2.2 Sample collection and pre-incubations

The peat used in this experiment were the combined remnants of samples previously taken from Exmoor National Park (see Section 3.2.2). However, as some of the peat and dominating *Sphagnum* moss cover had dried out during storage, it was essential to rehydrate the soil system and try to bring it back to 'natural' conditions. This was done via the creation of a 'bog bucket' where a *ca.* 20 L bucket was packed with the previously sampled peat – the sections of deeper peat (10–30 cm) at the bottom and surface peat (0–10 cm) sections placed on top – and filled with D.I. water until the surface was partially submerged. The bucket was then left outside in contact with sunlight and rain for approximately one month until the start of the experiment. As the bucket did not contain any holes for drainage, it was regularly checked and some water was poured out if there had been a lot of rain, to avoid overflow. After some time, both the peat and *Sphagnum* moss restored their moisture and colour. This process can be considered as a pre-incubation period to try to reinstate the original conditions of the peat as closely as possible.

For the replicate experiment later on (i.e. BB2), fieldwork was completed again at Exmoor National Park in early-March 2019 to collect more samples. Sampling was carried out at the same location and area of natural peatland as before. This time, however, only the surface layer of peat

(i.e. 0–10 cm) with rich *Sphagnum* coverage was taken. Within a few days after sampling, homogenised bulk peat (7 g) were subsampled into the incubation vials (30 mL). Bog water (3 mL) taken from the sampling site was transferred to the peat for additional moisture, before pre-incubating the vials under the set conditions (i.e. 25 °C and under 12 h intervals of light; see Section 4.2.3 below) for two nights. The top of the vial was covered with a pierced foil-lid to prevent further drying while still maintaining aerobic conditions. Incubations were then begun on the following day.

4.2.3 Incubations

Each 30 mL glass serum vial contained 7 g of gently homogenised surface layer peat and *Sphagnum* for the incubations. Again, the surface layer was selected based on research highlighting changes in the amount of N_2 fixed at different depths of peat (Warren *et al.*, 2017). Moreover, the turnover of organic matter is generally concentrated in surface soil and litter layers, making it more probable to be the horizon where the greatest N-fixing activity by free-living diazotrophs occurs (Jaiyebo and Moore, 1963).

D.I. water (3 mL) was added into each vial to help maintain moisture during the incubation period. Water addition serves another advantage by restricting the availability of O_2 in the soil system and supporting anaerobic conditions. An anaerobic indicator strip was placed inside, and the vials were tightly stoppered and sealed. The air inside was then evacuated and the system was put under vacuum.

A new 'artificial atmosphere' of $^{15}N_2$: O_2 (32 mL; gas mix with 76 % $^{15}N_2$ (98 % atom) in O_2; CK Isotopes, Leicestershire, UK) was subsequently injected into each incubation vial using a gas tight syringe (30 mL) with a fitted needle. Assuming that all the N_2 inside the vial is now made up entirely of $^{15}N_2$ (rather than occupying just 10 % of the vial headspace), incorporation of the ^{15}N-label into the soil microbial biomass should become much more pronounced – if any N-fixing activity occurs. The pressure in each vial was measured whilst waiting for the system to re-equilibrate back to atmospheric pressure.

The prepared energy source was introduced into the samples by injection. A long disposable hypodermic needle (2 3/4") was attached to a plastic syringe (1 mL) and used to draw up the Glc solution (180 µL). The needle was then carefully inserted through the rubber stopper on top of

the vial and pushed downwards until the tip of the needle touched the vial bottom. Finally, to attain uniform distribution, the needle was slowly withdrawn up through the peat soil as the plunger was depressed (Murphy *et al.*, 1999). This energy source was replenished after one week of incubations (i.e. day 8), by following the same injection method.

A CO_2 trap was fitted to every vial. All incubations were carried out at 25 °C underneath a growth light (12 h intervals). These conditions were set up on the basis of previous research. Studies have achieved higher levels of N_2 fixation when peat samples were exposed to light and *Sphagnum* were also seen to have turned a more vibrant green (Larmola *et al.*, 2014; Warren *et al.*, 2017). This suggests that the most active free-living diazotrophs in peat are either phototrophic organisms, or heterotrophic N-fixers fuelled by the carbohydrates provided via photosynthesis (Larmola *et al.*, 2014). Kravchenko and Doroshenko (2003) found that the optimum temperature for nitrogenase activity in peat was between 25–30 °C; while root nodules also revealed maximum activity within a similar temperature range (Trinick, 1980). A full summary of the refined incubation procedure that was followed is provided in Figure 4.2.

Incubations were all conducted triplicate, so there were three separate vials for each timepoint of the experiment. The experiments were halted in liquid N_2 after periods of 2, 4, 7, 10, 12 and 15 days and stored at -20 °C until freeze-drying. Freeze-dried peat soils were then finely ground using a mortar and pestle and stored in sealed vials until analysis.

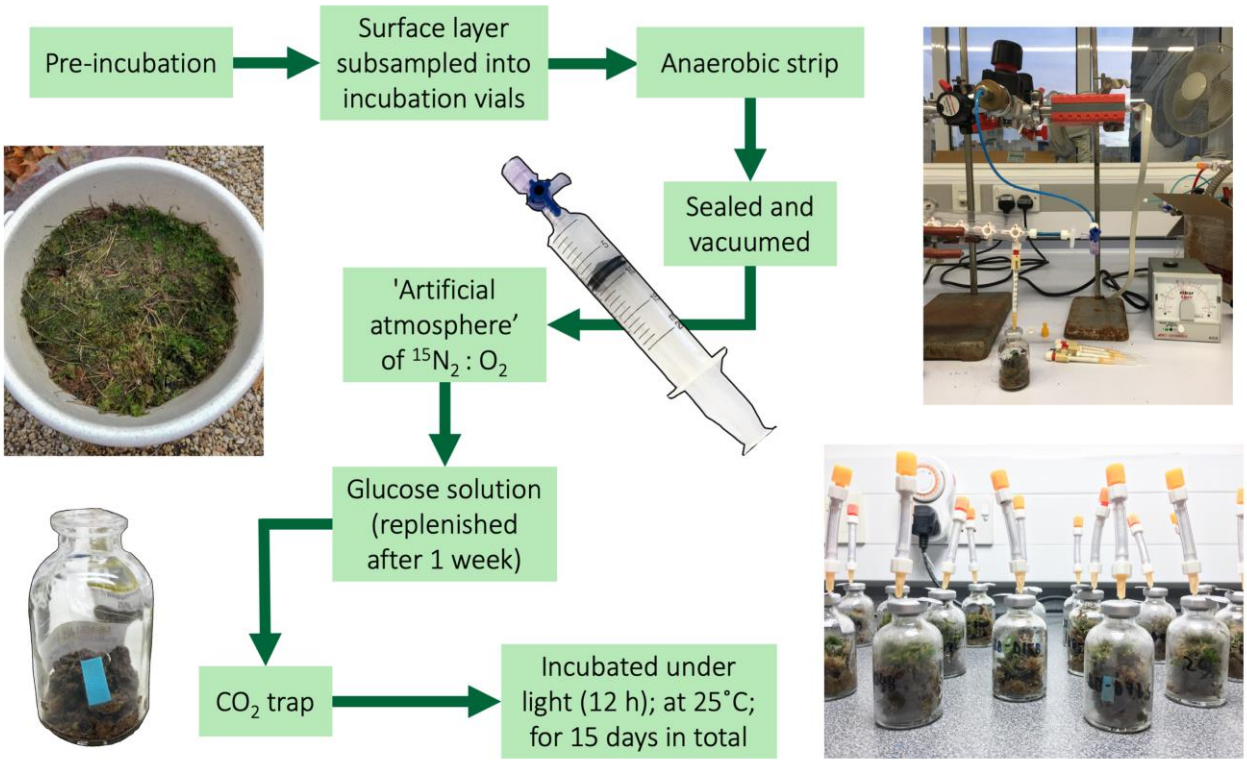


Figure 4.2. A complete overview of the refined experimental method, starting from the pre-incubation through to the incubation stage. Accompanying images illustrate specific processes including putting the sealed vial under vacuum (top right), and the final incubation set-up with a timed growth light (bottom right).

4.2.4 Instrumental analyses

Extraction protocols and AA analyses were carried out as described in Section 2.3, while the majority of the instrumental analyses can be found in Section 2.4. Data processing was as described in Section 2.5.

4.2.4.1 Elemental analysis: total nitrogen content

Bulk soil percentage total nitrogen (TN) and total carbon (TC) were attained using a Thermo EA1110 elemental analyser (Thermo Electron Corporation, MA, USA). Each sample of peat were weighed (*ca.* 10 mg) into a tin capsule and carefully sealed. The sample was then introduced singly into a combustion tube, set at 950 °C. Pure chromium oxide (Cr_2O_3; Elemtex, Cornwall, UK) and O_2 inside the tube were utilized as a combustion aid. A helium (He) carrier gas transported the combustion products over heated copper (Cu; Elemtex) wire for the reduction of any N oxides. Furthermore, silvered cobaltous/cobaltic oxide (Elemtex) ensured removal of

sulphur, while magnesium perchlorate ($Mg(ClO_4)_2$; Sigma-Aldrich, UK) was used to remove water. A GC column (60 °C) separated the resultant N_2 and CO_2, which were subsequently detected by a thermal conductivity detector (TCD). Data was collected and processed in Clarity (Version 2.6.2; DataApex, Prague, The Czech Republic). The % TN and TC of the soil samples were calculated based on a calibration standard of aspartic acid (CE Instruments Ltd., Wigan, UK) alongside a soil of known % TN and TC as the quality control (QC; NC Soil Std, CE Instruments Ltd.).

4.3 Results

4.3.1 Bulk $\delta^{15}N$ analyses, amino acid concentrations and total nitrogen concentrations

The addition of $^{15}N_2$ gas did not result in significant alterations to the soil's N status, as shown in the % TN values of the peat throughout the experiment (Table 4.1). No observable trend can be seen and the SEM of all the averages are small. It could be noted that the % TN on day 15 is slightly higher than on the previous days, though this may be attributed to small growths by *Sphagnum* during the long incubation – since both the moss and soil layer were taken together as the bulk peat. The total hydrolysable AA N content of the peat produced an average of 49.1 % (SEM 1.57) of the TN over the course of the incubations (Table 4.1). These concentrations all fit within the range of 20–60 % that is usually presented for total hydrolysable soil AAs (Kuzyakov, 1997; Schulten and Schnitzer, 1998; Senwo and Tabatabai, 1998; Bol *et al.*, 2004), and can also be equated to the concentration of the soil protein pool (Roberts and Jones, 2008).

Table 4.1. Average total hydrolysable AA (THAA) concentrations in peat (in mg AAs per g of dry peat) and the percentage contribution of THAA N to the total N (TN) pool.

	Time / days						
	0	2	7	10	15	**Mean**	**SEM**
Av % TN	1.25	1.19	1.11	1.22	1.35	1.22	0.033
Ala	3.93	4.44	3.79	4.11	4.76	4.21	0.176
Gly	3.36	4.31	4.10	4.32	4.46	4.11	0.197
Val	2.74	3.33	2.88	3.47	3.85	3.26	0.202
Leu	3.50	3.55	3.12	3.62	3.93	3.54	0.131
Ile	1.73	2.29	1.96	2.49	2.76	2.24	0.183
Thr	5.59	6.53	5.66	6.22	6.97	6.19	0.260
Ser	2.96	3.37	2.96	3.25	3.62	3.23	0.127
Pro	2.81	3.03	2.56	2.84	3.19	2.89	0.108
Asx	6.06	6.51	5.64	6.18	6.82	6.24	0.200
Glx	6.89	6.84	6.34	6.94	7.55	6.91	0.192
Hyp	0.46	0.49	0.52	0.53	0.60	0.52	0.023
Phe	1.47	1.45	1.24	1.46	1.66	1.46	0.067
Lys	1.88	2.92	2.76	2.32	3.10	2.59	0.220
Tyr	0.95	0.90	0.76	0.80	1.12	0.91	0.064
THAA	44.3	50.0	44.3	48.6	54.4	48.3	1.90
%THAA N of TN	43.2	52.5	50.3	49.4	50.3	49.1	1.57

Average concentration / mg g^{-1}

Following the injection of the ^{15}N-labelled gas, bulk soil δ^{15}N values showed a substantial overall increase over the course of the experiment (Fig. 4.3). At the start of the incubations, bulk soil δ^{15}N values remained relatively constant – displaying a minor peak on day 2. Major changes were observed from day 7 onwards, where the re-addition of Glc to samples on day 8 led to a prominent increase in δ^{15}N values. The system had also effectively gone anaerobic after roughly 7 days, proven by the anaerobic indicator strips inside each incubation vial (i.e. indicator strips fully changed to white after one week of incubation). A second replicate experiment, but with freshly collected surface peat (BB2; Fig. 4.3.), displayed even higher bulk δ^{15}N values and followed a similar trend in enrichment activity.

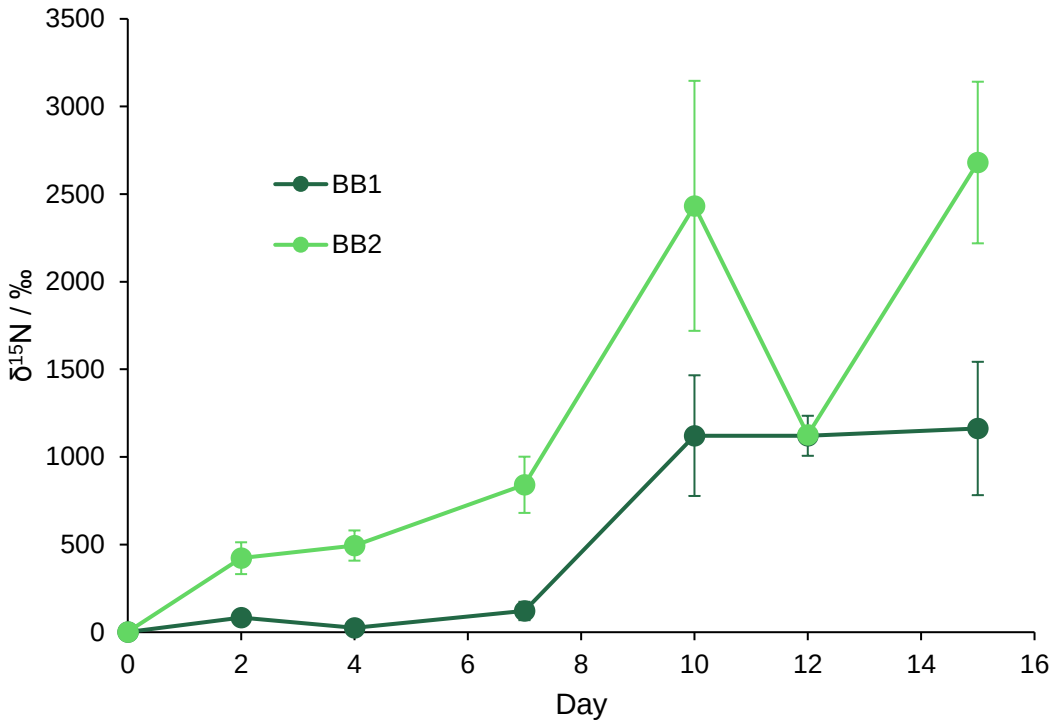


Figure 4.3. Mean bulk soil δ^{15}N values for the peat incubation experiments: the original experiment (BB1) is shown in dark green, while the subsequent repeat experiment (BB2) is in light green. Glc was first added on day 0 and re-supplied on day 8. Error bars are ± SEM (n=3).

4.3.2 GC-C-IRMS $\delta^{15}N$ values and percentage incorporations

Figure 4.4 shows the $\delta^{15}N$ values for individual hydrolysable AAs throughout the incubation experiment (for BB1 only). The trend observed from the bulk peat analyses is also reflected here, although the higher specificity of compound-specific GC-C-IRMS has allowed for even higher $\delta^{15}N$ values. Average percent ^{15}N incorporations were calculated based on the amount of ^{15}N applied and the amount incorporated into individual AAs, represented by Figures 4.5a and 4.5b, respectively. The percent applied ^{15}N incorporation is also equated to the percentage of ^{15}N fixed by diazotrophs within the peat, as measurable by the soil protein pool. When looking at the AAs individually, Glx displayed the highest incorporations for both percent applied and retained calculations (Fig. 4.5).

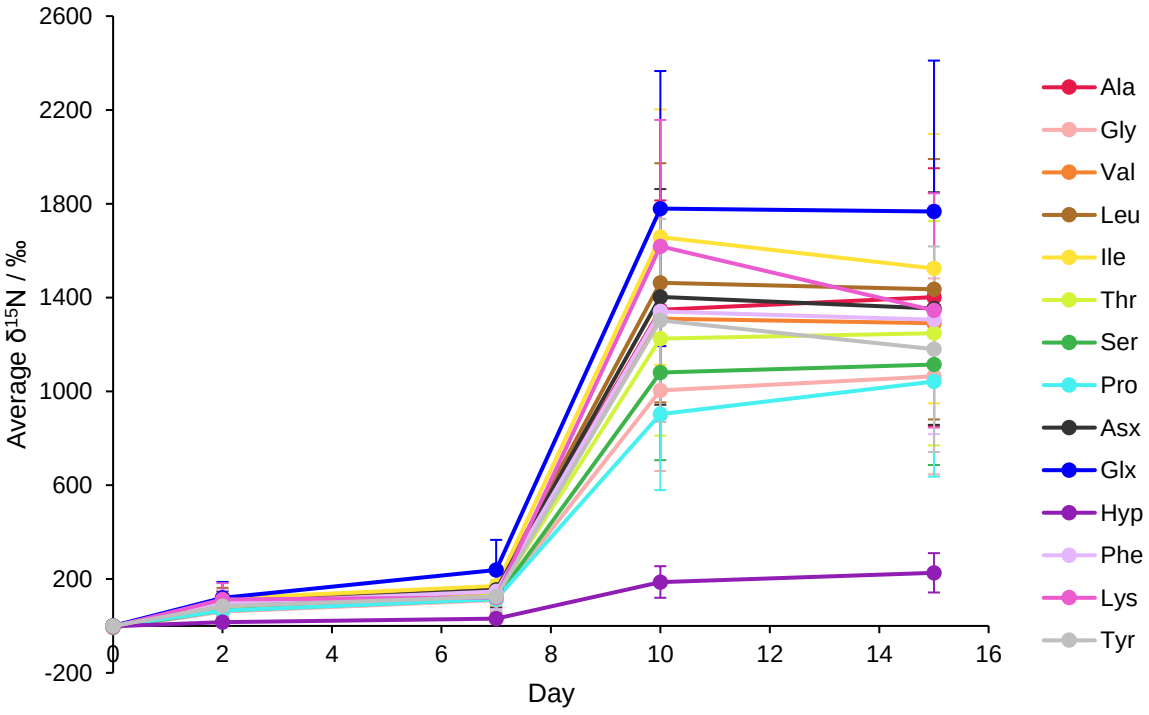
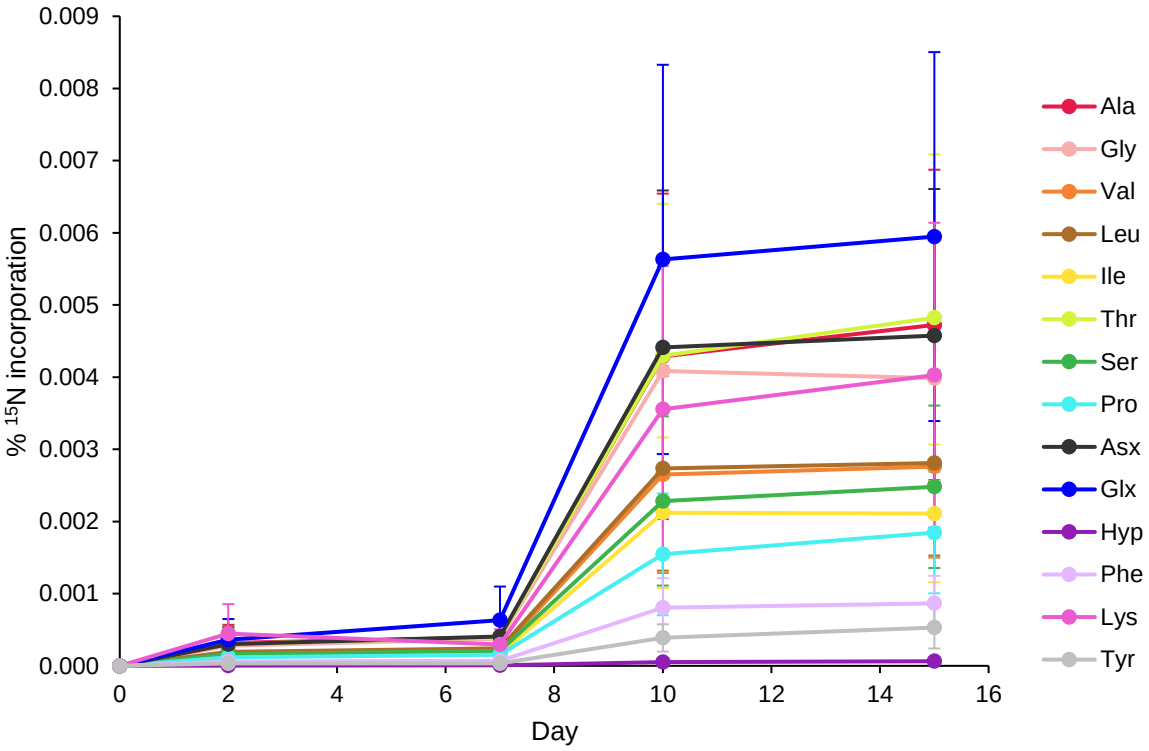


Figure 4.4. $\delta^{15}N$ values of individual AAs for the peat incubation experiment (BB1). Glc was first applied on day 0 and re-supplied on day 8. Error bars are ± SEM (n=3).

a) Applied (i.e. % Fixation)



b) Retained

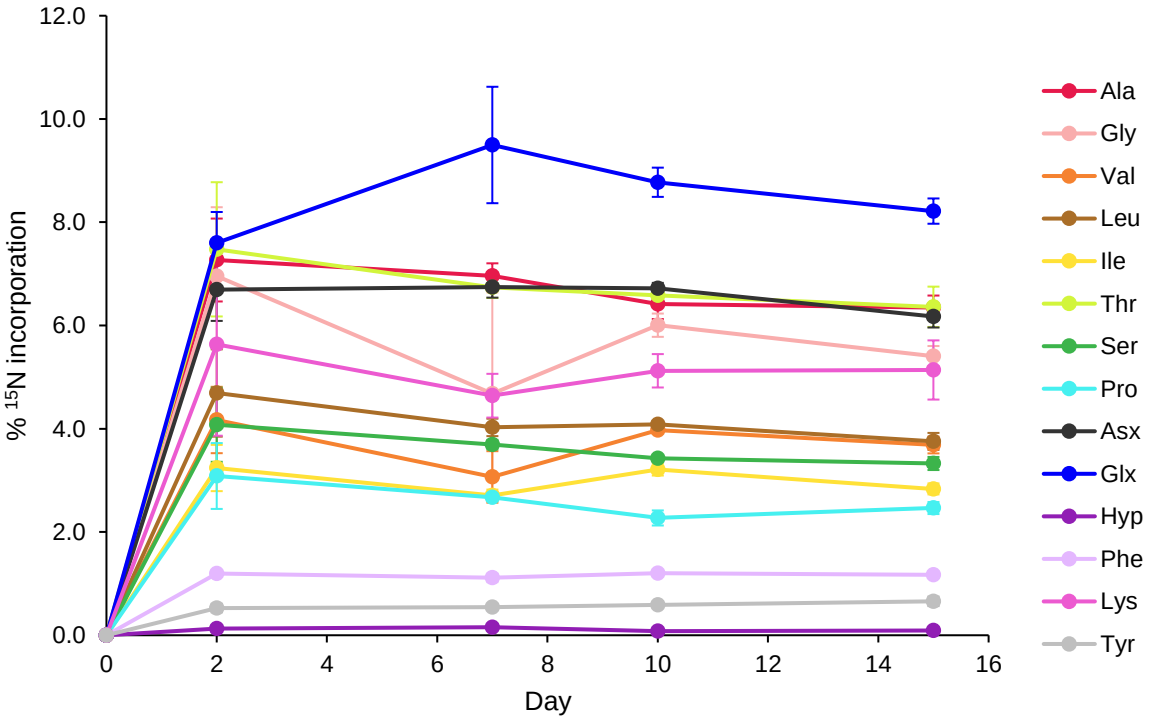


Figure 4.5. Average percentage ^{15}N incorporations into individual hydrolysable soil AAs over the course of the peat incubation experiment (BB1): a) applied – also referred to as percentage N fixed, and b) retained. Error bars are ± SEM (n=3).

4.4 Discussion

4.4.1 Evaluating the refined method and patterns in nitrogenase activity

Results suggest that anaerobicity and re-addition of the Glc solution were both very effective tools in promoting N_2 fixation by diazotrophs within the peat soil. The overall N-fixing activity of peat can supposedly be divided into two phases based on the observed trend. The first phase is where lower amounts of N_2 fixation occurred (i.e. day 0 to day 7), while the latter phase sees a significant climb in ^{15}N incorporation into the soil protein pool (Fig. 4.5a). Two major changes took place at the beginning of this second stage: the system had become fully anaerobic and energy was also replenished. This suggests that these factors were greatly influential in inducing the surge in nitrogenase activity and that refinements to the developed method were successful.

Moreover, the noticeable rise and fall in $\delta^{15}N$ values at the beginning of the incubations may be indicating the exhaustion of ATP energy within the microbial system (BB1, Fig. 4.3). The slight increase on day 7 further suggests that anaerobicity creates a more suitable environment for N_2 fixation, where subsequent energy replenishment on day 8 instantly spurred diazotrophic activity. This characteristic is much more evident in BB1, although this may be due to the fresh condition of the peat used in BB2. The observed pattern also coincides with results presented by Kravchenko and Doroshenko (2003) where a second addition of Glc resulted in immediate N_2 fixation activity (Fig. 4.1). Interestingly, the concentration of soil protein biomass in the period of maximum nitrogenase activity was similar to that before the second addition of Glc (Table 4.1). This suggests that Glc (or products of its oxidation) stimulate N_2 fixation by inducing enzyme synthesis or by activation of existing enzymes in the peat, rather than via an increase in the number of N-fixing bacteria (Kravchenko and Doroshenko, 2003).

The initial delay in N_2 fixation activity may stem from bacterial cells needing to biosynthesise or activate the nitrogenase enzyme, in order for it to become fully-functional (Kravchenko and Doroshenko, 2003). Free-living diazotrophs are known to exhibit a diauxic growth pattern, where there is a considerable lag before nitrogenase activity is noticed. The length of time taken for this catalytic enzyme to be biosynthesised varies depending on the type of organism, as well as its environmental conditions (Hill, 1992). Once biosynthesised, however, nitrogenase components may not be capable of instantly performing N_2 fixation. Protein maturation of the Fe and MoFe

protein must first be achieved. The actions of both *nif* and non-*nif* genes determine the rate of this step and allows nitrogenase to ultimately attain catalytic competency (Hill, 1992; Rubio and Ludden, 2005). Murphy (1975) also noticed a similar lag when Glc was added to a range of soils at 25 °C under anaerobic conditions. For most soils, the effect of this Glc addition was only apparent after 40 h into the incubation; for peat soil, this lag extended to 90 h. Thus, in our experiment, it may be that nitrogenase had only been effectively activated after the first phase of the incubation – and as the system became fully anaerobic – so that N_2 fixation could occur immediately after the second energy injection.

The large errors in N_2 fixation estimates likely reflect the high variability and spatial heterogeneity of these biological systems. Although each timepoint was conducted in triplicate, the incubations were carried out in three separate vials, and values therefore refer to three independent microbial systems. Consequently, some averages show more inconsistency – resulting in the relatively large errors on days 10 and 15 (Fig. 4.3, 4.4 and 4.5a). The rates of N_2 fixation are also expected to vary greatly within a landscape and there are likely to be 'hotspots' where high N-fixing activity occurs (Alexander and Schell, 1973; Reed *et al.*, 2010). Such hotspots can be merely centimetres apart from a non-hotspot (Reed *et al.*, 2010). These are a common phenomenon for non-symbiotic N_2 fixation as well as other microbially-mediated processes such as methanogenesis (Davidson *et al.*, 2004) and denitrification (Groffman *et al.*, 2009). While the causes of fixation hotspots are largely unknown, the size and frequency of these occurrences can have a strong effect on N_2 fixation estimates. Thus, even though the peat samples were carefully homogenised for the incubations, the community composition of free-living diazotrophs can differ profoundly even over very small spatial scales (Reed *et al.*, 2011).

4.4.2 So how much nitrogen does peat fix?

Average percentages of fixed N were also calculated based on the amount of ^{15}N measurable in bulk peat and the total N content of the dry peat (Table 4.2). At first glance, these values appear to be very small, reaching merely 0.073 % (SEM 0.053) by day 15. Similar values are expressed in Figure 4.5a, where the total percentage of fixed N measurable by all hydrolysable AAs, reaches a maximum of 0.0416 % (SEM 0.0191) after 15 days. However, these reported amounts must also take consideration of how much $^{15}N_2$ gas was added to the incubation vial and hence, soil system. The ^{15}N-enrichment of a given N pool is dependent on the amount of ^{15}N-label applied as well as the size of the N pool (Bedard-Haughn *et al.*, 2003). As all the 'natural air' was

evacuated from the vial and replaced with 76 % of $^{15}N_2$ at 98 % atom, a substantial amount of ^{15}N-label was present within the incubation system. Calculations of percent fixation are based on the total amount of ^{15}N applied to the peat and, therefore, the resulting incorporation %s appear small due to the large quantity of labelled material used.

Table 4.2. Average percent N_2 fixation, based on the amount of ^{15}N applied to bulk peat and the total N content of the peat. (Brackets are SEM; n=3).

Day	2	4	7	10	12	15
Average % N Fixation	0.005 (0.007)	0.001 (0.001)	0.006 (0.007)	0.067 (0.059)	0.074 (0.019)	0.073 (0.053)

This data can also be viewed from another perspective, by concentrating only on the number of moles of ^{15}N present within the peat samples. Figure 4.6 displays the amount of newly fixed ^{15}N per g of peat as a portion of the total amount of ^{15}N present in the bulk sample. By day 10, over 50 % of the total amount of ^{15}N present in the sample was made up of newly assimilated ^{15}N protein. This presents a more striking visualisation of the considerable level of ^{15}N incorporated into bulk peat as a result of the refined incubation method.

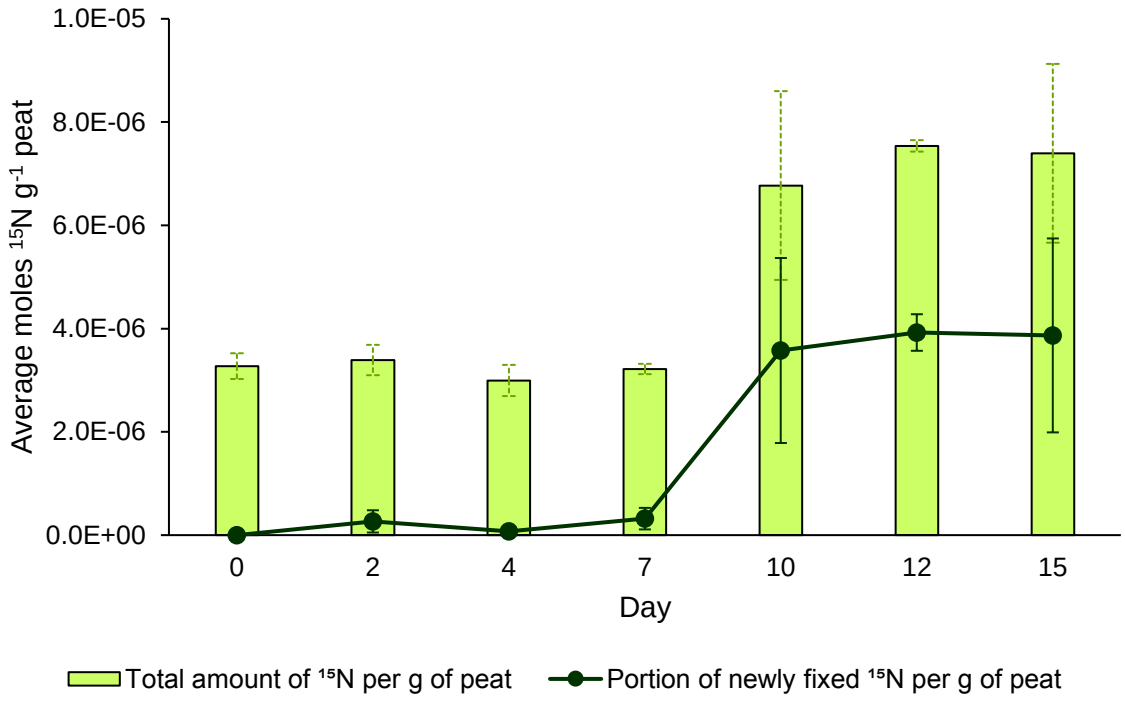


Figure 4.6. Average amount of N fixed (in mol ^{15}N per g of dry peat) as a portion of the total amount of ^{15}N in the bulk sample. Error bars are ± SEM (n=3).

Experimentally determined values of N_2 fixation were compared with those reported in the literature (Table 4.3). The results obtained match well with those available from existing studies of N_2 fixation by diazotrophs in peat from relatively similar sites and experimental conditions. More importantly, the developed method was able to measure even higher amounts of fixed N when compared to findings in literature. While Kravchenko and Doroshenko (2003) also supplied the peat with Glc, the samples were incubated under a gas mixture made up of 30 % $^{15}N_2$, 20 % O_2 and the remainder with Ar. Incubations were conducted in the dark and analyses were made on bulk soil. In their study, Larmola *et al.* (2014) conducted incubations under longer light conditions (20 h day length) and under headspace enrichment levels of 21 % $^{15}N_2$ – compared to 12 h day lengths and 76 % $^{15}N_2$ in our method. This confirms the effectiveness of our method in quantifying N_2 fixation in peat soil, while also highlighting the enhanced precision and sensitivity of compound-specific ^{15}N-SIP via GC-C-IRMS. Our results also demonstrate the significant potential of free-living diazotrophs to contribute to N_2 fixation in pristine peatlands.

Table 4.3. Comparison of experimental N_2 fixation values for peat to those found in literature. Sources: a) Kravchenko and Doroshenko, 2003 (peat from depth of 10–20 cm); b) Larmola *et al.*, 2014 (peat sampled from a fen-bog transition).

	Experimental value	Literature value
mg N fixed kg^{-1} dry peat / 15 days	57.94	8.13[a]
nmol N g^{-1} dry peat / 15 days	3868	2160[b]

4.4.3 Correlations between the amount of fixation and the diazotroph community in peatlands

Amounts of N_2 fixation measured through this refined method also agrees with reports on the abundance of nitrogenase genes present in peatlands. As previously covered in Chapter 3, low-pH soils of high organic matter and moisture content are said to possess more N-fixing genes and may therefore demonstrate greater fixation activity (Malik *et al.*, 2017). The high levels of ^{15}N enrichment detected as a result of the peat incubations (Fig. 4.3 and 4.4) strongly resonates this claim and provides new estimates of dynamic N_2 fixation in peatlands (Table 4.3).

N_2 fixation genes were also discovered to be most abundant in the top layer of peat collected from a *Sphagnum*-dominated, ombrotrophic and acidic bog (Lin *et al.*, 2014). At the same location, Warren *et al.* (2017) measured 1.2 x 10^7 copies of *nifH* g^{-1} in surface peat (1 and 20 cm) and only 0.2 x 10^7 *nifH* copies g^{-1} in the deeper layers (30 and 75 cm). Drawing on the fact that this site possessed similar conditions to our sampling site in Exmoor, it may be tentatively assumed that – under the right anaerobic and energetic conditions – a larger population of *nifH* genes does support higher N-fixing activity.

4.4.4 *Sphagnum* as a driver of nitrogen fixation in peatlands

It is rather unclear from this experiment whether N_2 fixation is primarily led by non-symbiotic N-fixers located in *Sphagnum* or in the peat soil itself. Many studies have exclusively reported on free-living N_2 fixation associated with *Sphagnum* or other native peat bryophytes (Berg *et al.*, 2013; Larmola *et al.*, 2014; Vile *et al.*, 2014; Leppänen *et al.*, 2015; Kox *et al.*, 2016). Many bog ecosystems are ombrotrophic in character, thus the only external sources of nutrients are rainwater and air. As the moss species has no direct contact with the soil and are without xylem or other nutrients transport system, microbes are therefore believed to play an essential role in the supply of N for *Sphagnum* mosses. They harbour a specific population of bacteria which are adapted to these unique conditions and carry out important functions for the moss – including the transfer of fixed N (Opelt *et al.*, 2007). Thus, *Sphagna* appear to accommodate a diverse diazotrophic community (Vile *et al.*, 2014; Knorr *et al.*, 2015; Leppänen *et al.*, 2015; Kox *et al.*, 2016); although, the true benefits for the microorganisms themselves are less evident (Kox *et al.*, 2018). Based on our developed method, a possible next step would be to isolate live moss from the peat soil and conduct the $^{15}N_2$ incubations and subsequent analyses on the separated plants. This would allow more concrete comparisons of each component's individual N-fixing capabilities – to see whether N_2 fixation is more active in live *Sphagnum* or the mass of partially decomposed organic matter beneath.

4.4.5 Pathways of nitrogen assimilation following fixation by diazotrophs in peat

In Figure 4.5, assimilation of fixed ^{15}N into Glx is noticeably higher than into any other AA. This trend of ^{15}N incorporation may be related to the fundamental biosynthetic pathways of N assimilation used by microorganisms. Glu is the main AA produced in the N assimilation pathway by N-fixing bacteria (Eq. 4.1; Nagatani *et al.*, 1971; Miflin and Lea, 1976b), serving as

an important N-transporter and N-donor in the biosynthesis of other compounds (Santero *et al.*, 2012). It is biosynthesised by glutamine synthetase (GS) in conjunction with glutamate synthase (GOGAT), where N must first react to form Glu before being converted into various other AAs (Miflin and Lea, 1976a). Nagatani *et al.* (1971) also found a wide distribution of GS within free-living diazotrophs. Moreover, Glu production is reportedly induced by both light and C sources – when photosynthesis takes place and C skeletons are in abundance, N is increasingly assimilated and transported as Glu (Lam *et al.*, 1995). Thus, this provides a possible explanation for the elevated levels for Glx seen over the incubation period (Fig. 4.5). The identification of such trends also emphasises the additional need to conduct compound-specific ^{15}N isotopic analysis via GC-C-IRMS. By investigating newly biosynthesised AAs, the assimilation dynamics of fixed N into the soil organic N pool can be followed and further insights regarding the microbial pathways following N_2 fixation can be gained.

$$NH_4^+ + \alpha\text{–oxoglutarate} + ATP + NADPH \leftrightharpoons \text{glutamate} + ADP + P_i + NADP^+$$

Equation 4.1. The main pathway of N assimilation, catalysed by glutamine synthetase (GS) and glutamate synthase (GOGAT).

5 Investigating different sources of ATP and their effect on free-living nitrogen-fixers in British grassland soils

Now that the developed method was successfully implemented on peat, it was important to conduct further evaluations using other soil types as well. Compared to natural ecosystems, the managed aspect and relative simplicity of agricultural systems provide a great opportunity to investigate the functioning of diazotroph communities in soil. In this regard, agricultural experiments conducted over a long-term are especially beneficial, given that it can take an extended period of time for the microbial community structure to equilibrate with respect to alterations in management practices (Hsu and Buckley, 2009). Accordingly, this chapter will apply our refined procedure to study N_2 fixation in a range of British grassland soils collected from three different long-term experimental fields.

It was also previously concluded that free-living BNF in soils is largely limited by the lack of available energy sources, where significant N_2 fixation was stimulated by Glc additions. The particular energy source, however, is not only limited to Glc. Due to intense competition for such supplies, non-symbiotic diazotrophs are able to utilise a wide range of simple organic compounds for ATP (Jensen, 1965). In this chapter, we compare the effectiveness of different C sources in facilitating N_2 fixation by addition of an artificial root exudate mix to the soil incubations.

5.1 Root exudates and the rhizosphere

The rhizosphere is the volume of soil around plant roots which is influenced by root processes and is consequently a zone of generally high microbial activity (Hinsinger *et al.*, 2009). In the rhizosphere, the observed elevation in density and activity of microorganisms that is clearly distinct from bulk soil is fuelled by the release of C-rich substances known as root exudates (Baudoin *et al.*, 2003). These are diffusible compounds that are reflexively excreted by the plant root into surrounding soil, over which they have minimal direct control (Jones *et al.*, 2009). Root exudates (RE) are complex mixtures of water-soluble, low molecular weight substances typically consisting of free sugars (e.g. Glc, sucrose), AAs (e.g. Gly, Glu) and organic acids (e.g. malic acid, oxalic acid) (Kraffczyk *et al.*, 1984; Jones *et al.*, 2009; Mus *et al.*, 2016). These reflect the dominant compounds central to plant cell metabolism such that the composition and amount of

RE released are highly variable between species and environments (Hertenberger *et al.*, 2002; Jones *et al.*, 2009; Mommer *et al.*, 2016). Studies have indicated that a large percentage (64–86 %) of RE released were quickly respired by the microbial population (Hütsch *et al.*, 2002). Thus, the rhizosphere environment is distinguished by a profusion of readily available C (Kuzyakov, 2002), which can influence the soil microbial community structure and cycling of key nutrients such as N (Mus *et al.*, 2016).

Accordingly, BNF is commonly heightened in rhizosphere soil, compared to bulk soil, owing to the availability of labile C as an energy source for free-living N-fixers (Lovell *et al.*, 2000; Bürgmann *et al.*, 2005). This may also provide reasoning for the high diazotroph activity detected in surface peat (Chapter 4). Energy provided by RE are easily assimilable without the biosynthesis of extracellular enzymes, making these exudates a further source of available C that are likely to be sought out by microorganisms (Baudoin *et al.*, 2003). However, as the rhizosphere contains large quantities of both N-fixing and non-fixing microbes, there may be considerable competition for the acquisition and use of this C supply (Yoneyama *et al.*, 2017). A key question driving our investigation is thus 'how effective are RE as an energy source for diazotrophs in fixing N_2?'

5.2 Effect of soil management practices on N_2 fixation

Different soil treatment practices can influence the structure of the diazotrophic community and their corresponding N-fixing activity due to changes in plant composition as well as the type and amount of fertiliser applied (Mäder *et al.*, 2002). Coelho *et al.* (2008) discovered that the presence of low levels of N fertiliser increased the abundance of free-living diazotrophs by 30 % compared to higher N levels. Crop rotations were the dominant management factor found to produce a higher diversity of N-fixing bacteria in a study by Orr *et al.* (2011). Nevertheless, while these studies help provide a greater understanding of the microbial community composition, they do not necessarily say anything about the function. There is still ambiguity regarding how long-term field applications of fertiliser or organic (i.e. legume or manure) N sources affect diazotrophy in the soil.

For this experiment, it is hypothesized that agricultural soils treated with N fertiliser or other organic inputs of mineral N (i.e. NH_4^+ and NO_3^-) will administer less N_2 fixation relative to unfertilised soils or those from more pristine ecosystems. Preceding evidence from both laboratory and field studies indicate that when fixed N is already available in the environment,

diazotrophs can favourably utilise it rather than performing fixation themselves (Reed *et al.*, 2011). Diazotrophs to satisfy their N demands through means other than N_2 fixation. These microorganisms may also acquire mineral N externally from the environment or internally via the enzymatic breakdown and relocation of cellular N (Hill, 1992; Reed *et al.*, 2011). Thus, free-living diazotrophs can deactivate N_2 fixation when mineral forms of N are easily accessible in the environment (DeLuca *et al.*, 1996). On the other hand, when external stores of mineral N in the soil are depleted, the low availability of N can stimulate BNF (Reed *et al.*, 2011).

5.3 Site descriptions and sample preparations

Different grassland soils for the incubation experiments were sampled from three separate locations. A basic map of the different sampling sites is shown in Figure 5.1. Table 5.1 summarises the different soils and the individual treatments they have received.

Table 5.1. A summary of the soils used for the incubation experiments in this chapter.

Sample Name	Site / Affiliation	Original Treatment and Usage
BNGR	Henfaes Research Centre, Bangor University	Previously received inorganic N fertiliser and lime; used for year-round grazing and silage production
NW	Middle Wyke Moor, North Wyke Farm Platform	Increased use of legumes; cattle and sheep grazing
PG	Park Grass Experiment, Rothamsted Research	Three separate treatments: i) nil-treated (i.e. received no N fertiliser), ii) N applied as NH_4^+, and iii) N applied as NO_3^-.

5.3.1 Henfaes Research Centre

The first type of soil was taken from Henfaes Research Centre in Aberwyngregyn, Wales (53°14'0.18"N, 4° 0'59.91"W), managed by Bangor University. The field site was situated in the lowlands of the research station and has an average altitude of 12.1 m. It is a semi-permanent sheep-grazed grassland dominated by *Lolium perenne* L. grass and serves year-round grazing at a moderate stocking density (2–3 livestock units per hectare). The site has also been used for silage production as of April 2009. The soil was classified as a freely draining Eutric Cambisol (FAO) and has a sandy loam texture. Inorganic N fertiliser had previously been applied to the field at rates of 100–130 kg N ha^{-1} y^{-1}, together with phosphorus (P) and potassium (K). Liming

was also carried out to uphold a pH of 6.5 (Reay, 2019). The top soil (0–15 cm) was collected and sieved to 5 mm.

5.3.2 North Wyke Farm Platform

Another soil was sampled from Middle Wyke Moor sub-catchment at the North Wyke (NW) Farm Platform in Okehampton, Devon, UK (50°45'49.21"N, 3°53'50.92"W). The Farm Platform receives steady amounts of precipitation annually, which is distinctive of major agricultural grasslands in Southwest England. Hence, the environmental conditions are enough to sustain 280 days of grass growth each year – though livestock grazing season is often limited to 180 days due to soil wetness. Beginning in April 2013, alternative types of management were progressively implemented on certain farmlets. Middle Wyke Moor was treated with an increased use of legumes where the AberHerald (*Trifolium repens* L.) white clover cultivar was introduced and mixed with AberMagic (*Lolium perenne* L.) grass (Wu *et al.*, 2016). The soil is a pelo-stagnogley soil (a clayey non-calcareous soil) of the Hallsworth series (British Classification). Soils were sampled between the depth of 0–15 cm from the surface.

As the collected soils were very clayey and wet, the samples were pooled together and slightly dried out in preparation for the incubation experiments as carried out by Charteris (2016). Briefly, tweezers were used to gently divide moist clumps which were spread out in foil-lined trays and left to air-dry. The adequately dried soil was passed through a clean 2 mm sieve and then stored in a glass jar fitted with a punctured foil-lid to prevent over-drying, whilst still sustaining aerobic conditions. The process was conducted with great care to avoid smearing and compressing the soil with the purpose of preventing substantial destruction of the soil microbial community.

5.3.3. Park Grass Experiment

The last type of soil was acquired from the Park Grass Experiment (PGE) at Rothamsted Research in Harpenden, Hertfordshire (51°48'14.40"N, 0°22'22.80"W). The uniqueness of this site is that its plots have received nearly the same annual supply of nutrients (N, K, P, Mg, Na and Si) through inorganic fertilisers since 1856 – and is designated as the longest-running experiment in the world. Soil from this site is classified as a Chromic Luvisol (FAO) and sample cores (10 x 10 cm) were collected from three sub-plots comprised of three different N

management histories: i) a nil-treated plot which receives no fertiliser N (i.e. control plot), ii) a plot where fertiliser N is applied as NH_4^+, and iii) a third plot where N is applied as NO_3^-. Lime was also applied to all three sub-plots to maintain an approximate pH of 6. Samples were air-dried and sieved to 2 mm before storage (following the same protocol mentioned previously). Further details on the site, sampling method and preparations can be found in Dunn (2017).

5.4 Experimental methods

5.4.1 Preparation of the artificial root exudate mix

To investigate the effects of different sources of ATP on free-living N-fixers, an artificial RE solution was prepared. Sucrose (Fisher Scientific, Loughborough, UK), glucose, fructose (Alfa Aesar, Heysham, UK) and malic acid (Acros Organics, Morris Plains, NJ, USA) were dissolved in DDW to provide a solution containing 10 mg C mL^{-1} (adapted from Griffiths *et al.* (1999); excluded succinic acid and N-containing compounds). This concentration is the same as the formerly prepared Glc-only solution (Section 4.2.1). The compounds chosen represent those commonly reported to appear in RE (Griffiths *et al.*, 1999). Sucrose, Glc, and fructose are the main sugars known to facilitate heterotrophic growth, whereas malic acid can act as a specialised C transporter between plants and bacteroids (Mus *et al.*, 2016).

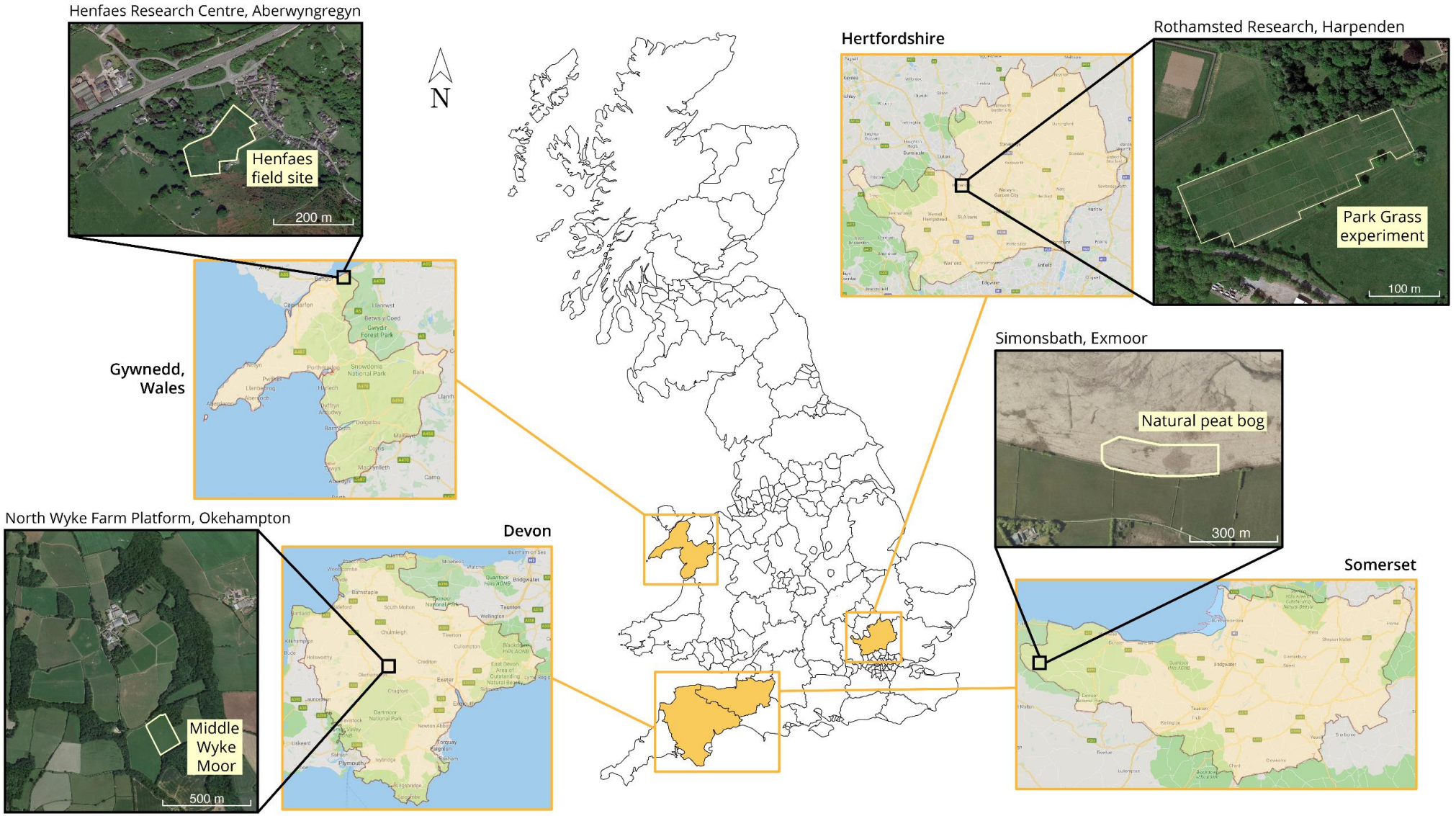


Figure 5.1. Map showing the location of all the different soil sampling sites within the U.K. © 2019 Google. © 2019 Getmapping plc.

5.4.2 Pre-incubations

Each soil was separately homogenised and weighed into 30 mL glass serum vials (7 g). D.I. water (3 mL) was added to each subsample for additional moisture, before covering the vial with a punctured foil-lid to prevent further drying. All vials were then left to pre-incubate under the established conditions (i.e. at 25 °C and under 12 h intervals of light) for one week prior to adding the ^{15}N-label. Effects from long-term storage and soil disturbances may have affected the soil microbial community – where sieving, for example, is known to increase rates of mineralisation (Jones and Shannon, 1999). Thus, pre-incubating soils under the incubation conditions can allow microbes to equilibrate to the new conditions before undergoing further procedures.

5.4.3 Incubations

Following the incubation procedure described in Section 4.2.3, incubations were carried out in triplicate and over periods of 0, 3 and 15 days. Additionally, the RE mix was also added (180 μL) to soils from the Henfaes and NW sites in order to compare the effect of different energy sources on the N_2 fixation process. A set of vials was injected with Glc only (10 mg C mL^{-1}; 180 μL), on days 0 and 8, while another set received energy purely from the artificial RE mix on those same days.

5.4.4 Instrumental analyses

Extraction protocols and instrumental analyses were carried out as described in Section 2.3 and Section 2.4. Based on results from bulk δ^{15}N analyses, subsequent AA analyses for compound-specific data was only conducted on control and final-day incubation samples (i.e. day 0 and day 15). Data processing was as described in Section 2.5.

5.5 Results and discussion

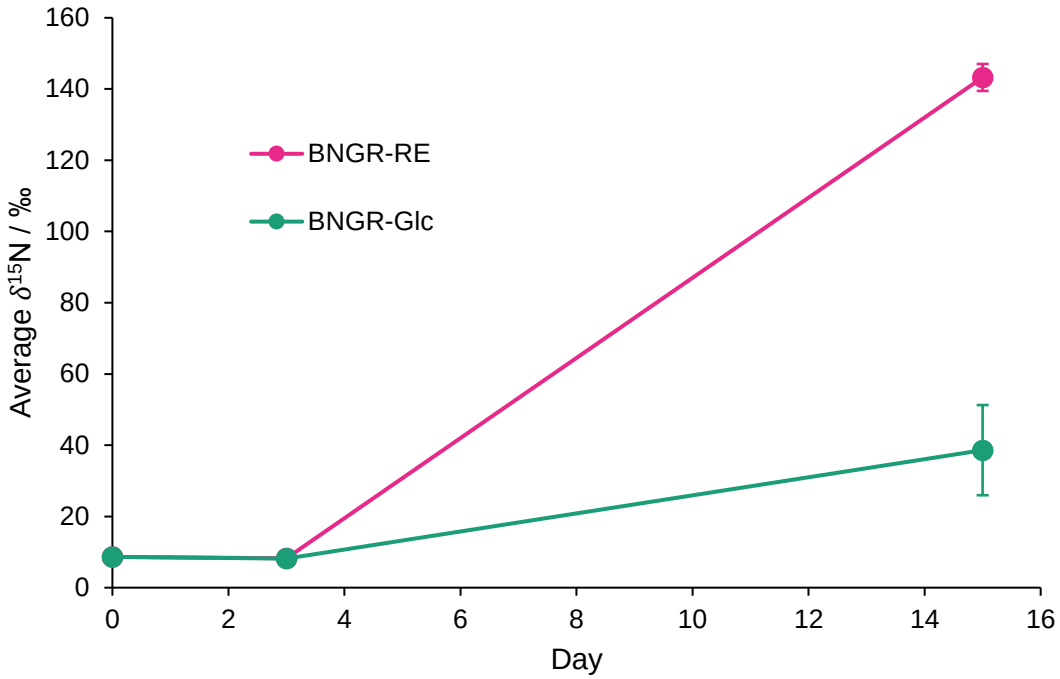
5.5.1 Limitations of bulk $\delta^{15}N$ analyses in detecting nitrogen fixation in grassland soils

Average bulk soil $\delta^{15}N$ values revealed no significant changes in enrichment between control and day 15 samples for most soil types (Table 5.2). Soil from the Henfaes site showed the highest enrichment, where average $\delta^{15}N$ values went from 8.63 ‰ on day 0 to 38.61 and 143.25 ‰ on the final day for samples injected with Glc only and samples injected with a RE mix, respectively (Table 5.2; Fig. 5.2). On the other hand, soils from the NW site showed very limited N-fixing activity and there were no apparent differences in bulk $\delta^{15}N$ values with regards to the source of energy applied (i.e. Glc *vs.* RE). N_2 fixation appeared to be unfavourable in all three treatments from the PGE, revealing no detectable enrichment via bulk ^{15}N analyses (Table 5.2).

Conversely, data provided by compound-specific ^{15}N-SIP using GC-C-IRMS strongly showcases the higher sensitivity of the method compared to bulk techniques. The raw $\delta^{15}N$ values of the hydrolysable soil AAs were higher than those detected from bulk soil analyses (Fig. 5.3 and 5.4). For example, the Henfaes soils supplied with a RE mix reached a $\delta^{15}N$ value of over 400 ‰ on day 15 (Fig. 5.3b). Figures 5.5 and 5.6 presents the mean percentage applied ^{15}N incorporations (i.e. % N fixed) into individual hydrolysable soil AAs between control and final-day incubations for all the grassland soils. Although the amount of fixation on day 15 is very slight, a general increase in the incorporation of ^{15}N into most AAs can be observed. This detail was not possible through bulk ^{15}N analyses, especially with the soils from NW and PG. The advantages of compound-specific ^{15}N-SIP via GC-C-IRMS are even more apparent in Figure 5.7. By combining the % applied ^{15}N incorporations of all hydrolysable soil AAs, the resulting total % proves that N_2 fixation was actually taking place across the different soil types. Except for the NH_4^+-treated soil from PG, the rest all showed total increases in the amount of ^{15}N fixed into the soil protein pool. This exemplifies how highly sensitive the compound-specific GC-C-IRMS technique is, in its ability to detect low levels of diazotrophic activity that could not be attained from bulk soil.

Left: **Table 5.2.** Mean bulk soil $\delta^{15}N$ values for all soil types and treatments. Right: **Figure 5.2.** Mean bulk soil $\delta^{15}N$ values for the Henfaes soil incubation experiment. Energy was supplied on days 0 and 8: soils which received only Glc is shown in dark green, while the RE mix is shown in pink. Error bars are ± SEM (n=3).

Sample Name	Day	$\delta^{15}N$	SEM
BNGR-Glc	0	8.63	0.09
	3	8.16	0.13
	15	38.61	12.66
BNGR-RE	0	8.63	0.09
	3	8.24	0.05
	15	143.25	3.78
NW-Glc	0	6.71	0.04
	3	5.93	0.07
	15	7.28	0.26
NW-RE	0	6.71	0.04
	3	5.62	0.03
	15	7.31	0.16
PG-NIL	0	3.00	0.04
	3	3.15	0.13
	15	3.18	0.14
$PG\text{-}NO_3$	0	3.81	0.07
	3	3.10	0.04
	15	3.58	0.42
$PG\text{-}NH_4$	0	2.63	0.07
	3	2.04	0.00
	15	2.44	0.19



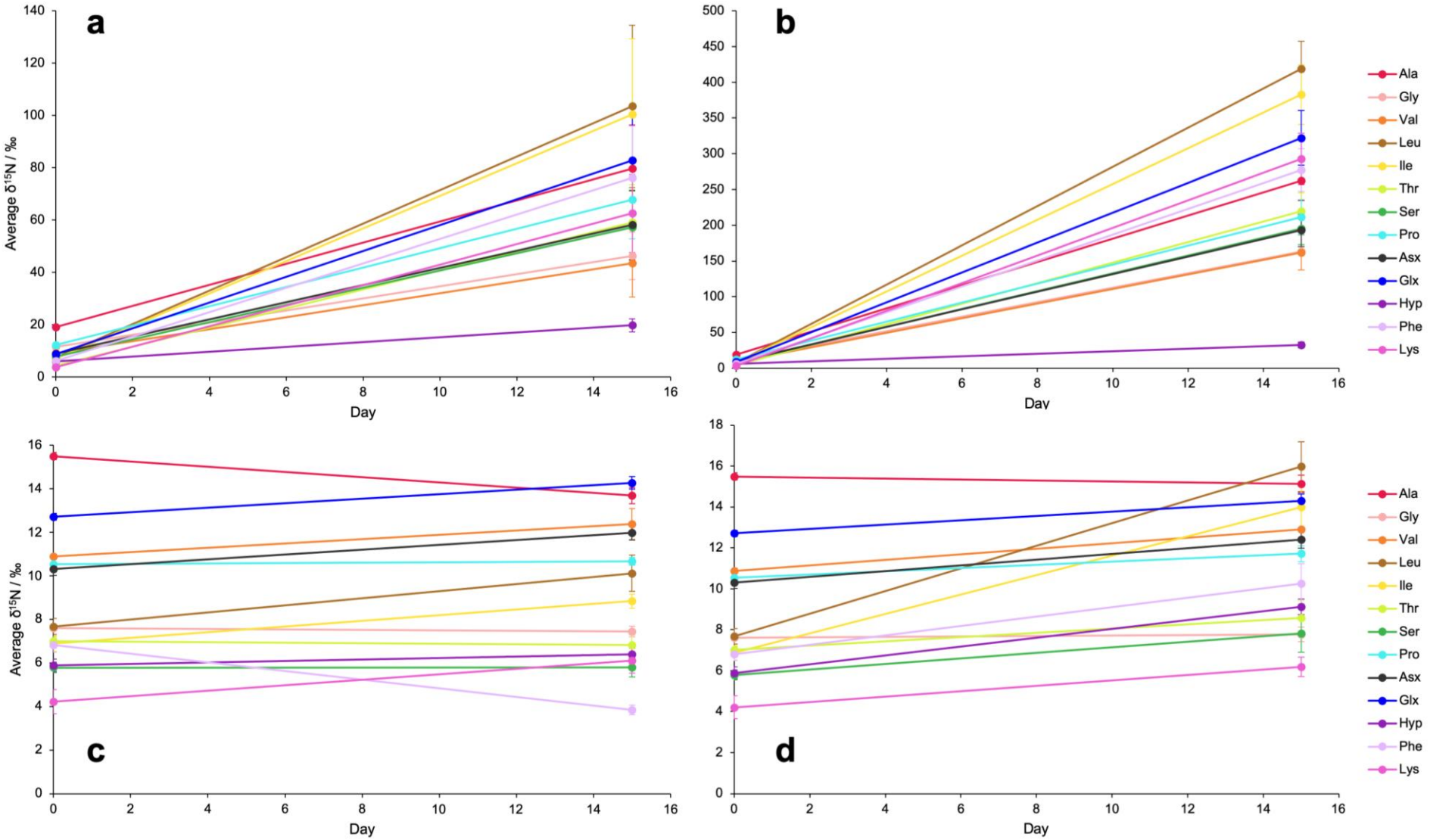


Figure 5.3. Average $\delta^{15}N$ values of individual AAs between control and day 15 samples for Henfaes and North Wyke soils, which received either Glc or a RE mix as an energy source: a) BNGR-Glc, b) BNGR-RE, c) NW-Glc and d) NW-RE. Error bars are ± SEM (n=3).

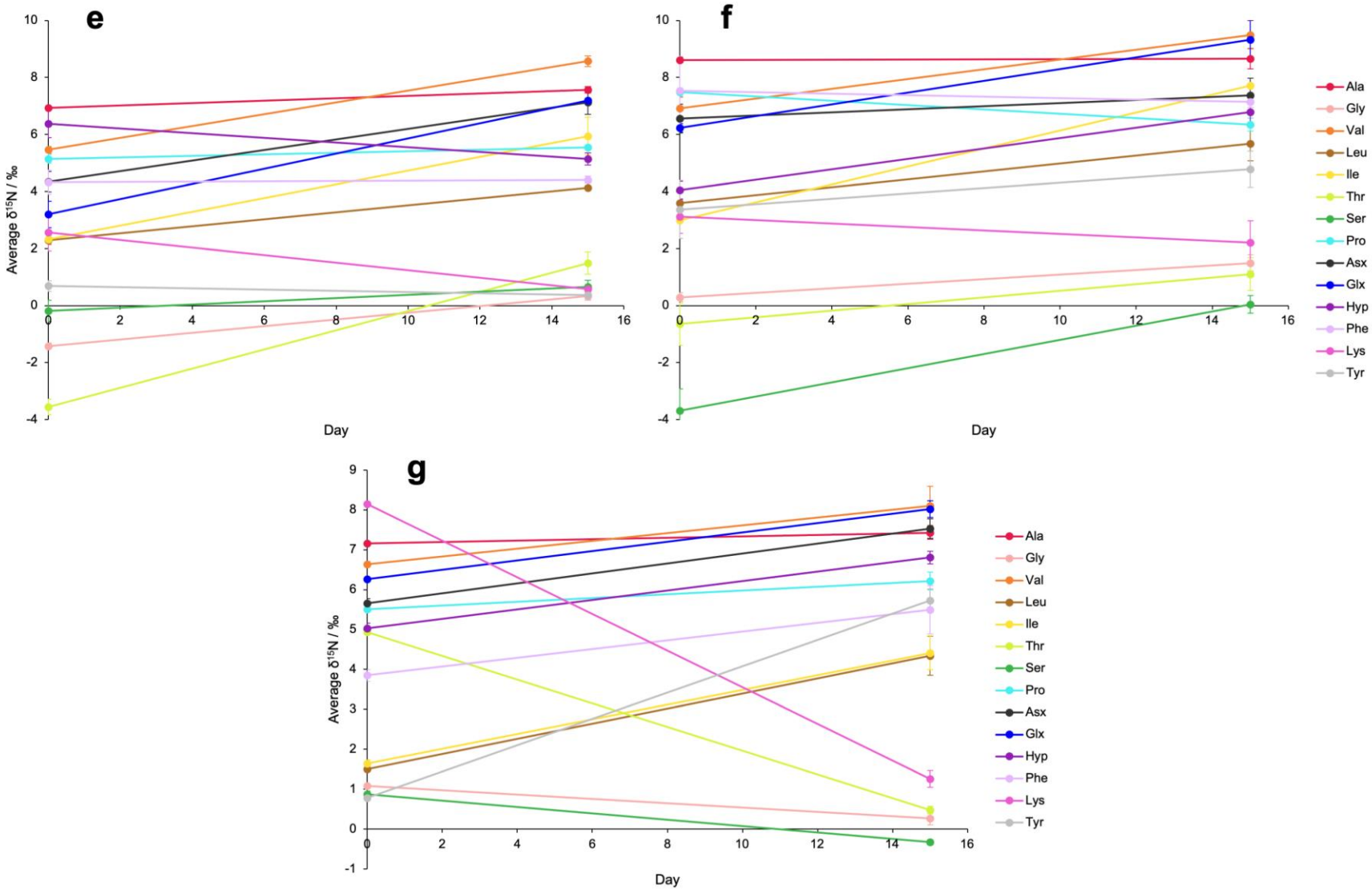


Figure 5.4. Average $\delta^{15}N$ values of individual AAs between control and day 15 samples for each Park Grass soil treatment, which only received Glc as an energy supply: e) NIL treated, f) NO_3^-, g) NH_4^+. Error bars are ± SEM (n=3).

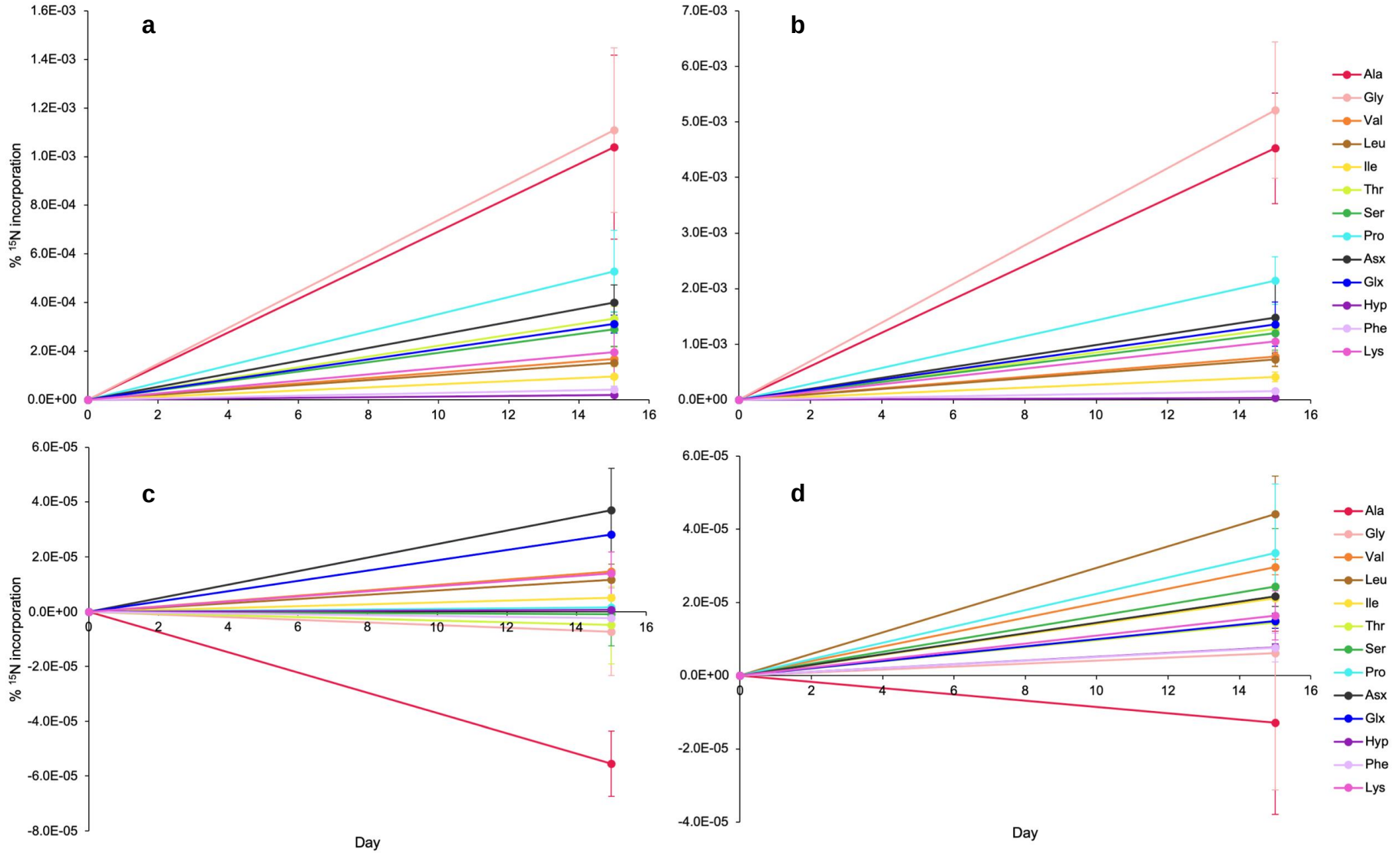


Figure 5.5. Average percentage applied ^{15}N incorporations (i.e. % N fixed) into individual hydrolysable soil AAs between control and day 15 samples for each soil type, which received either Glc or a RE mix as an energy source: a) BNGR-Glc, b) BNGR-RE, c) NW-Glc and d) NW-RE. Error bars are ± SEM (n=3).

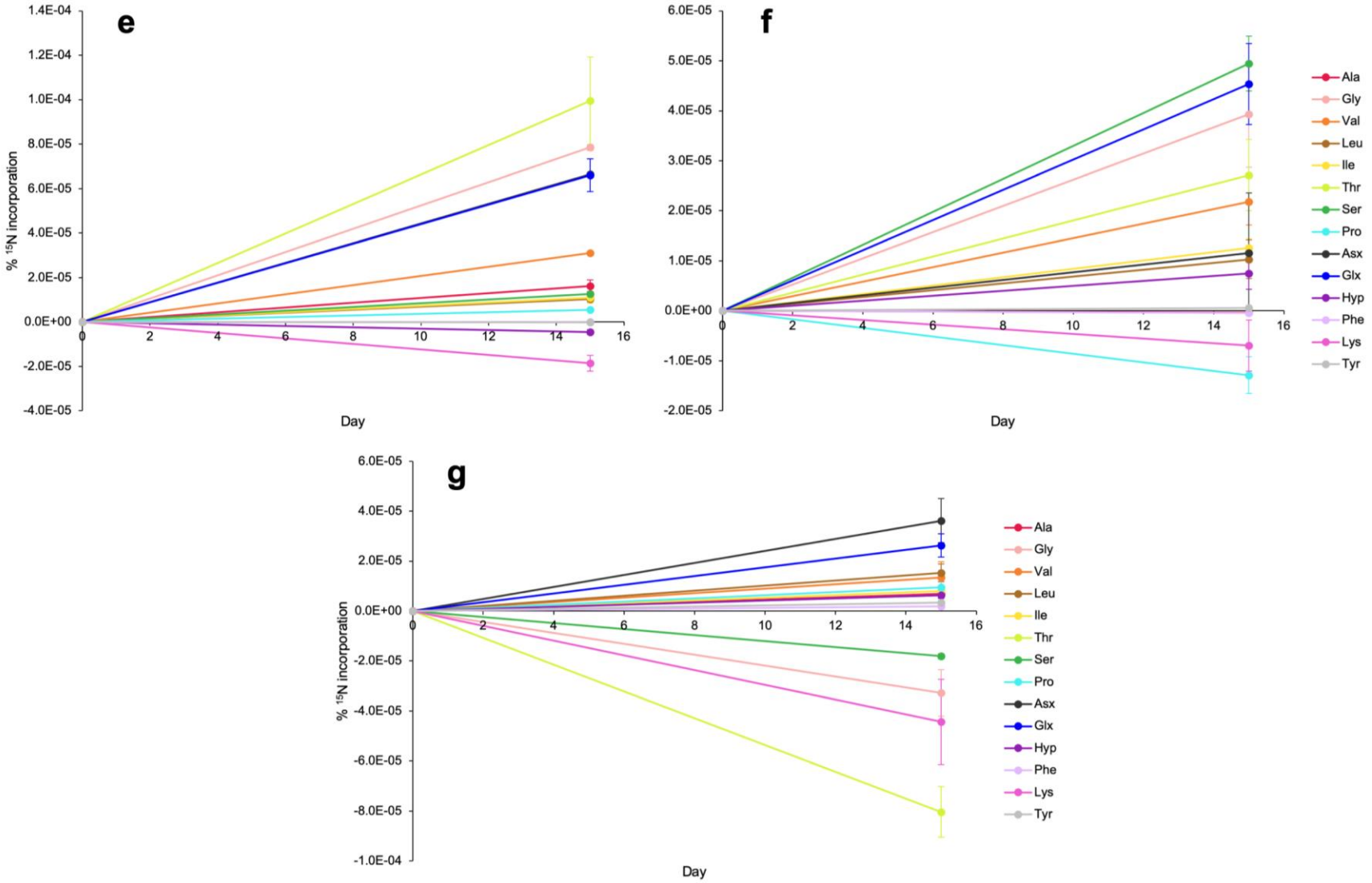


Figure 5.6. Average percentage applied ^{15}N incorporations (i.e. % N fixed) into individual hydrolysable soil AAs between control and day 15 samples for each Park Grass soil treatment, which only received Glc as an energy supply: e) NIL treated, f) NO_3^-, g) NH_4^+. Error bars are ± SEM (n=3).

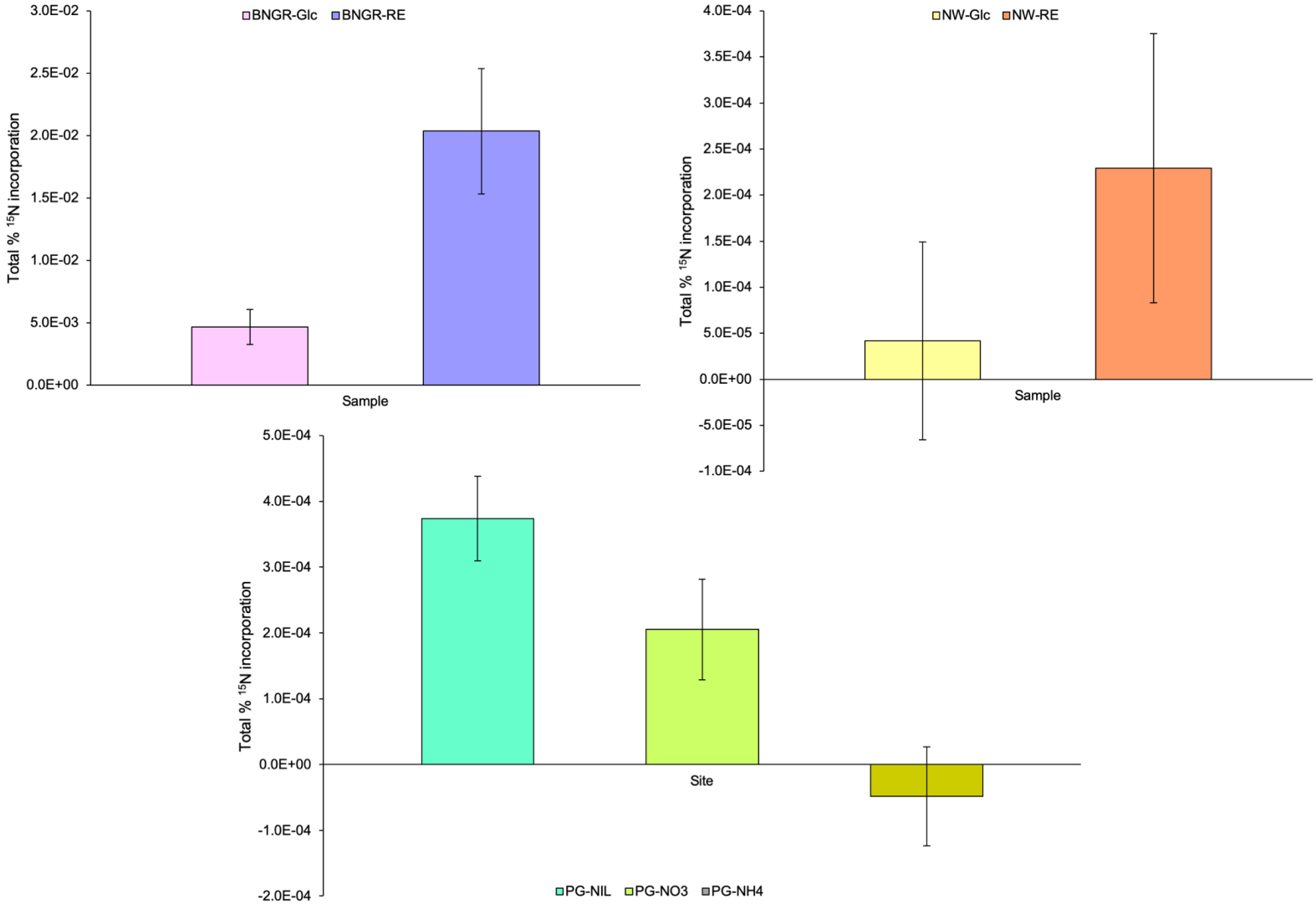


Figure 5.7. Total percentage applied ^{15}N incorporated into all hydrolysable soil AAs for different soil types and treatments. Error bars are ± SEM (n=3).

5.5.2 Effectiveness of the root exudate mix as an energy source

The data presented in Figures 5.2 and 5.7 both suggest that the RE mix is a more effective source of energy for free-living diazotrophs to fix N_2, when compared to Glc exclusively. Both substrates were applied at the same C concentration on soils from Henfaes and NW. Both sites showed greater levels of ^{15}N incorporation when RE were used as an energy source. In particular, for the Henfaes soil, the level of $\delta^{15}N$ enrichment increased by over 3 times of the Glc-only incubations (Table 5.2; Fig. 5.2). Differences in N_2 fixation with respect to sources of energy could be ascribed to either fluctuations in active N-fixers and their distinct properties or the availability of the C substrate and breakdown products (Rao, 1978).

While microbial population and activity in the soil biomass is often limited by the availability C substrates, different diazotrophs may have different preferences in their C and energy sources. *Azospirillum*, for example, reportedly prefer organic acids as their C source – where certain strains such as *A. lipoferum* are unable to take up Glc. Free-living *Azotobacter*, however, can exploit a wide variety of sugars, organic acids and alcohols (Hill, 1992). Therefore, aside from environmental regulators such as O_2, the efficiency of N_2 fixation also relies on the particular source of energy used (Hill, 1992; Reed *et al.*, 2011). Soil microbial biomass has also been shown to increase with higher-diversity RE treatments as substrates are easily accessible by more microorganisms (Steinauer *et al.*, 2016). Accordingly, the diversity of compounds in a RE mix increases the chances of meeting those C preferences – especially when experimenting on an unknown diazotrophic community in the soil microbial pool.

Nevertheless, our developed method also presents some limitations. The quantity of C used (i.e. *ca.* 10 mg C g^{-1} soil) is rather unrealistic in relation to C concentrations estimated for rhizosphere soil. The amount of C entering the rhizosphere daily is calculated at around 50–100 mg C g^{-1} soil – though this value is not representative of all environments and ecosystems (Baudoin *et al.*, 2003). Moreover, the energy source was applied in a single pulse, which does not truly simulate the natural diffusion of RE. Taken together, these factors may underestimate the soil microbial system's full N-fixing capabilities; however, our developed method has paved the way for a more precise and mechanistic approach in investigating BNF and free-living diazotrophs involved in the process.

5.5.3 Availability of nitrogen in the environment and its impact on nitrogen-fixing activities

Grassland soils generally displayed lower levels of N_2 fixation, despite incubating the top soil layer and supplying an external energy source to the microbial system. Compared to natural peat which incorporated a mean total of 0.0416 % (SEM 0.0191) ^{15}N after 15 days, the highest total ^{15}N incorporation here was 0.0204 % (SEM 0.0050) by the Henfaes soil supplied with RE (Fig. 5.7). However, this difference in fixation values was expected for agricultural systems due to the availability of inorganic N from fertilisers and other management practices.

As the only unfertilised soil in this experiment, the nil-treated plot from PG was expected to show the highest rate of N_2 fixation – though this was not the case. Bulk ^{15}N analyses of the soil was unable to detect any changes in enrichment (Table 5.2), while GC-C-IRMS analyses averaged a total ^{15}N incorporation of merely 0.00037 % (SEM 6.4E-05; Fig. 5.7). These results are most likely caused by another source of inorganic N entering the soil, such that the 'unfertilised' plot has actually been provided with a stable supply of N over a prolonged duration. A probable candidate for this other source is atmospheric N deposition. Anthropogenic activities have led to a three-fold increase in N deposition globally (Reay *et al.*, 2008), where it is a major cause of soil acidification across various ecosystems (Goulding *et al.*, 1998). Storkey *et al.* (2015) reported that the amount of inorganic N deposited onto surface soil at the PGE is *ca.* 21 kg ha^{-1} yr^{-1} (between 2010–2012), which is approximately 1/5 of that administered as fertiliser to the plots (Dunn, 2017). Accordingly, the low levels of BNF witnessed in the nil-treated plot is not so surprising when considering alternative inputs of inorganic N.

Another explanation for the lower levels of N-fixing activity in grassland soils is the influence of livestock grazing, which applies to the Henfaes and NW fields. A large proportion of the N ingested in herbage by animals is returned unevenly to the soil through their urine and excrement. This results in a large spatial variability of the redistributed N (Vinther, 1998). Inorganic N released from animal waste also reduces legume N_2 fixation by fostering grass growth and thus lessening the proportion of these symbiotic N-fixers (Ledgard *et al.*, 2001). Vinther (1998) estimated that N_2 fixation in a grass-clover sward exposed to excreta – like the NW site – would be reduced by 10–15 %, compared to an un-grazed pasture. This therefore provides some reasoning for the low levels of fixation seen by the NW site.

5.5.4 Comparing experimental nitrogen fixation rates with available genetic insights

Studies on diazotroph community structures also mirrored N_2 fixation rates quantified using our developed method. Figure 5.8 depicts variations in *nifH* gene abundance at different soil depths for the Henfaes field site. Highest *nifH* abundances were detected in the top soil layer, which also showed high N-fixing activity as measurable through our method (Fig. 5.7). Most interestingly, the amount of *nif* genes in different treatment plots at PG also coordinates with our quantifications of N_2 fixation for the same site. In Figure 5.9, the low abundance of N-fixing genes overall is consistent with the low level of ^{15}N incorporation found across all PG plots – where *nif* genes and fixation both decreased in order of NIL > NO_3^- > NH_4^+ treatments. Altogether, these results indicate that N_2 fixation rates alter as a function of the diazotroph community and that these two components are well interlinked.

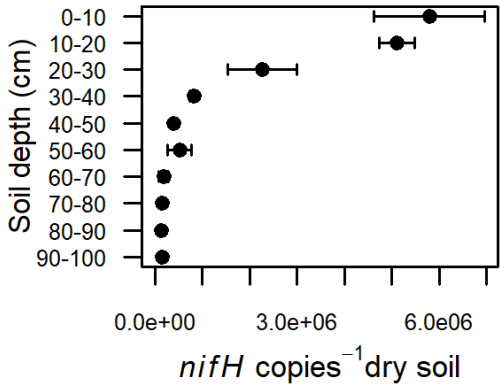


Figure 5.8. Variation in *nifH* gene abundance (copies number g^{-1} dry soil) as a function of soil depth for the Henfaes field site. Error bars are ± SEM (n=4). (Jones, 2019, unpublished data).

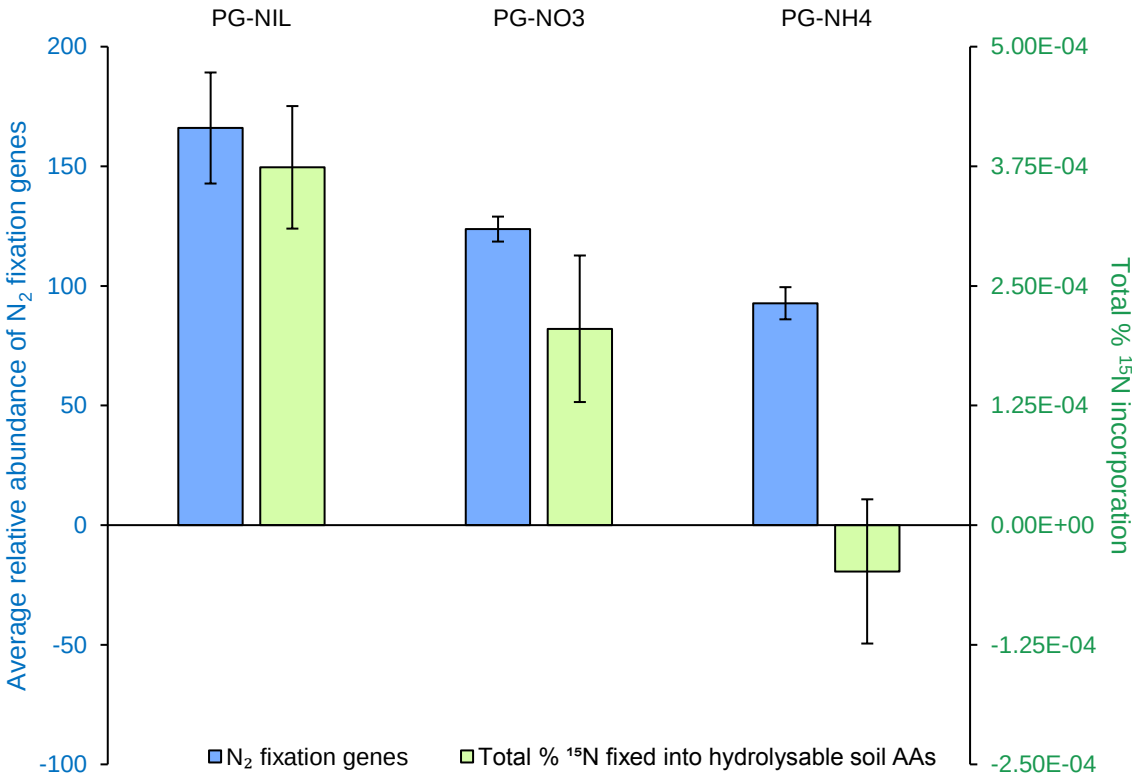


Figure 5.9. The abundance of N_2 fixation genes in different pH 7 N-treated plots at Park Grass (in blue) and the corresponding total % of ^{15}N fixed into hydrolysable soil AAs (in green). Genetic data was obtained from Griffiths (2019, unpublished data). Metagenome sequences were provided by Illumina Hiseq (NERC Sequencing Facility, Liverpool) and data was annotated using standalone SEED servers. The data was resampled so that each sample possessed the same number of reads, hence the values obtained are therefore relative abundances.

6 Final method validation: Working with soils from the Karst Critical Zone in southwest China

Soil degradation is a pervasive issue throughout China that is causing major constraints to achieving food security, where a sustainable increase in agricultural production is vital to provide for the rising population. At the termination of the 20th Century, China instigated its 'Grain for Green' programme by recommending the complete retirement of eroded farmland to allow for progressive recovery through the restoration of natural vegetation (Li *et al.*, 2018). This was also implemented in the Karst Critical Zone (CZ) of southwest China which has suffered from several decades of intensive agriculture, resulting in widespread landscape degradation and areas where soil function was essentially lost (Green *et al.*, 2019). The effects of this policy on soil N cycling is, however, still poorly understood (Li *et al.*, 2018). Thus, in this chapter, our developed method was extended to assay the Chinese karst landscape by quantifying N_2 fixation in soils at various stages of recovery. A more comprehensive understanding of the responses of free-living diazotrophs in recovering ecosystems can help to inform further strategies for sustainable soil conservation and management.

6.1 Karst landscapes in southwest China

The karst landscape of southwest China is among the world's largest, covering *ca.* 550,00 km^2 (Liang *et al.*, 2015). Its unique characteristics derive from the dissolution of carbonate bedrocks such as limestone and dolomite by acidic water from rainfall (He *et al.*, 2008). Due to its base geology, soil formation in the karst area is slow (Song *et al.*, 2017). The steeply sloping topography is variably scattered with thin overlying soil that is prone to erosion; the solubility of carbonate rocks also give rise to higher dissolution rates and fast percolation, causing many areas to experience surface water shortages (Wang *et al.*, 2004). There is also a significant potential for soil nutrient loss during heavy rainfall or the monsoon season due to significant runoff (Song *et al.*, 2017). As a result, ecological systems in southwest China's karst region are fragile and exceedingly susceptible to human disturbance. Rapid intensification of agriculture over the last 50 years has instigated extensive land degradation and desertification; the restoration of ecosystem integrity is therefore a prime concern of this region (Liang *et al.*, 2015).

The consequences of ecosystem degradation on soil microbial communities are to a great extent, still unknown – especially within karst landscapes. Most significantly, the application of both organic and inorganic fertilisers has had profound effects on the soil N cycle. BNF has been under intense scrutiny regarding the responses of diazotrophs towards the implementation of progressive natural vegetation recovery (Li *et al.*, 2018). Thus, recovering karst systems in southwest China offer a valuable opportunity to understand the effects of these actions. It is hypothesised that BNF will increase going up the recovery gradient; the increase in natural vegetation should lead to higher organic inputs from plant litter and roots and generate a more diverse soil microbial community.

6.2 Site descriptions and sample preparation

The study sites in this chapter were located in the Karst Critical Zone in Puding County, Guizhou Province, southwest China (Fig. 6.1). The area is predominantly underlain by limestone bedrock (Yang *et al.*, 2016). This is also reflected in the calcareous soils, which is classified as a Mollic Inceptisol (USDA Soil Taxonomy) (Li *et al.*, 2018). A subtropical monsoon climate covers the region, where rainfall largely ensues between May and October (Peng and Wang, 2012). Within the area, the main locations for sampling were Chenqi catchment (26°15'49.62"N, 105°46'18.97"E) and Tianlong Mountain (26°14'48.00"N, 105°45'51.00"E). Encircled by four hills, the catchment is a characteristic closed karst depression, with an elevation of 1316–1500 m above sea level (asl) (Peng and Wang, 2012). Approximately 2 km away from Chenqi, Tianlong Mountain has an altitude of 1460 m asl and is almost entirely covered by natural forests (Hao *et al.*, 2019).

Six levels of soil disturbances were selected to fully investigate the chronological effects of vegetative recovery on BNF (Table 6.1). In ascending order of disturbance, these included soils from: i) a sloping farmland, ii) a recently abandoned cropland (*ca.* 3 years), iii) an abandoned cropland of *ca.* 5 years, iv) an upslope long-term abandoned cropland (> 5 years), v) a higher upslope secondary forest which had previously been an abandoned pear orchard, and vi), a primary forest containing pristine natural vegetation. Soils were collected between April to June 2017. Terraced farmland in Chenqi catchment (referred to as 'Penny's Pit' (PP); 26°15'45.93"N, 105°46'39.67"E) is cultivated through crop rotation, generally consisting of maize, soybean and rapeseed. The plot receives an annual fertiliser N input of 225–375 kg ha^{-1} and also minor *ad hoc* inputs of human and livestock excreta. Crop residues are removed after the first harvest season and soils are then levelled through tillage for the second cropping (Li *et al.*, 2018). Incubations

were conducted on surface soil (i.e. O/A horizon) for TL and PP, whereas horizons were homogenised for all the CQ samples.

Table 6.1. A summary of the soil types studied in this chapter. (Description of vegetation communities taken from Li *et al.*, 2018).

Location	Sample Name	Land use / recovery phase	Vegetation
Tianlong Mountain	TL	Primary forest; undisturbed vegetation	Mature trees and understory species
Chenqi catchment	CQ-N1	Higher upslope; secondary forest (abandoned pear orchard)	Small trees and woody shrubs
	CQ-N2	Upslope; long-term abandoned terraced cropland (> 5 years, rotation crop)	Grasses and herbaceous species
	CQ-N3	Valley; abandoned cropland (*ca.* 5 years, rotation crop)	
	CQ-N4	Valley; abandoned cropland (*ca.* 3 years, rotation crop)	
	PP	Terraced/sloping farmland (rotation crop)	Agricultural crops

6.3 Experimental methods and instrumental analyses

Each soil was individually sampled into glass vials and pre-incubated for one week exactly as described in Section 5.4.2. Incubations were carried out in triplicate for 0, 3 and 15 days, following the developed protocol in Section 4.2.3. Based on results from the previous experiment on grassland soils, the artificial RE mix was used as the sole energy source (10 mg C mL^{-1}; 180 μL; Section 5.4.1).

AA hydrolyses for compound-specific analyses were only conducted on control and final-day incubation samples (i.e. day 0 and day 15) in accordance with the protocol in Section 2.3. Instrumental analyses and data processing were as described in Section 2.4 and Section 2.5, respectively.

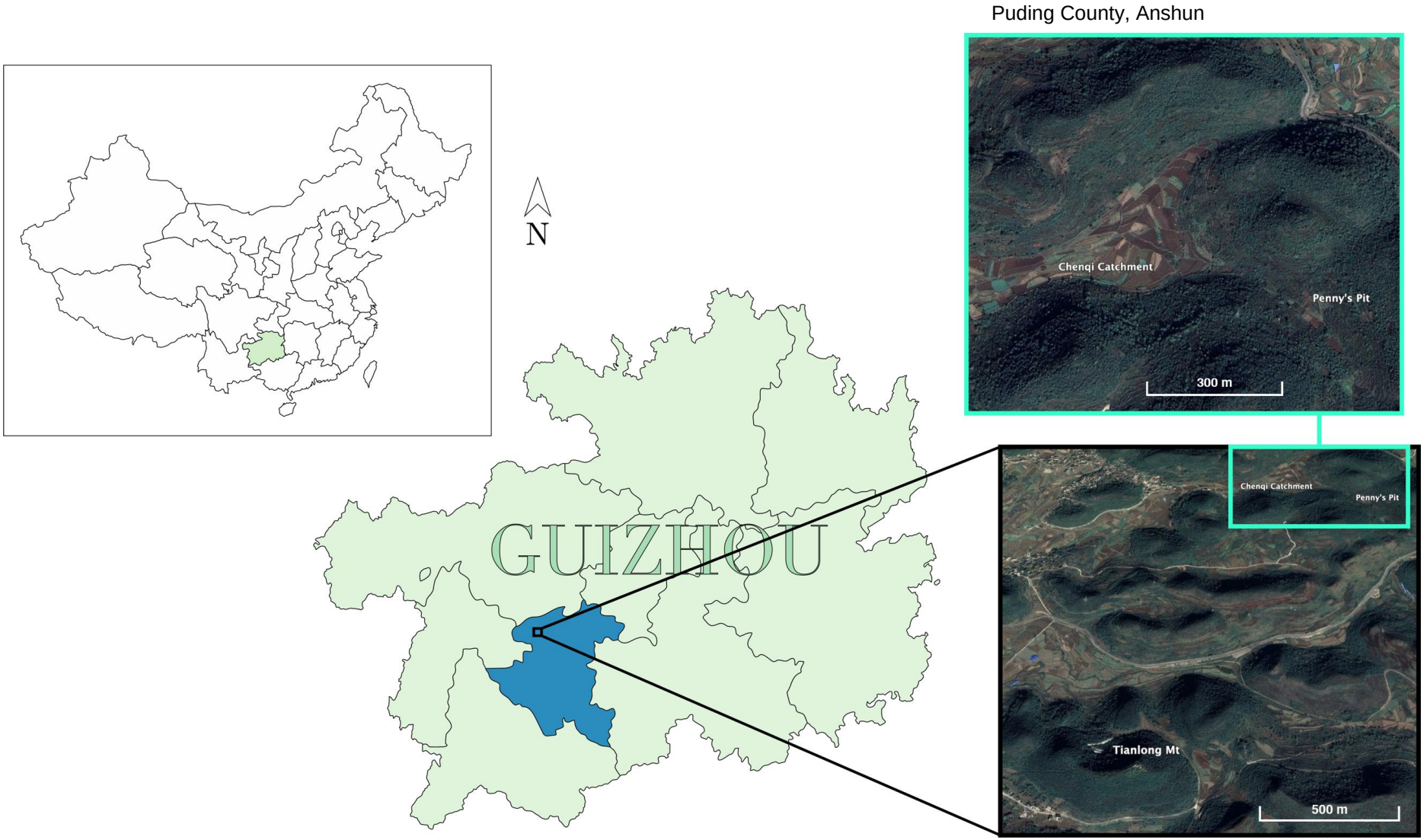


Figure 6.1. Map showing the locations of the SPECTRA sampling sites in the Puding Karst Critical Zone. © 2019 CNES / Airbus. © 2019 Maxar Technologies.

6.4 Results and discussion

6.4.1 Assessing nitrogen fixation in Chinese karst soils under progressive recovery stages

As hypothesised, the amount of N fixed by free-living diazotrophs within the karst soils generally increased and we moved up the land recovery gradient. Bulk soil $\delta^{15}N$ values showed high enrichment for all the CQ samples by day 15, reaching a highest average value of 675 ‰ for the secondary forest soil and around 457 ‰ for the 3-year abandoned cropland (Fig. 6.2). As expected, soil from the terraced cropland showed much lower enrichment – reaching a similar level to the Henfaes soil discussed in the previous chapter.

Figures 6.3 and 6.4 show the raw $\delta^{15}N$ values of the hydrolysable soil AAs for each of the karst soils. Due to the selectivity and lack of dilution from background soil N, $\delta^{15}N$ values were much higher compared to the bulk soil analyses – going over 3000 ‰ on day 15 for the CQ-N3 site (Fig. 6.3c). Compound-specific ^{15}N-SIP analyses via GC-C-IRMS also allowed for the amount of ^{15}N fixed into individual hydrolysable AAs to be calculated (Fig. 6.5 and 6.6). Except for the TL samples, increases in the incorporation of ^{15}N into most AAs were observed. A clearer depiction of the trend can be seen in Figure 6.7 where the total % of ^{15}N fixed into all hydrolysable AAs decreases with shorter soil recovery phases, going from: secondary forest > long-term abandoned cropland > *ca.* 5-year abandoned > *ca.* 3-year abandoned > terraced cropland. Due to its divergent results, the primary forest soil is excluded from this trend and is discussed later as an anomaly.

Enhanced SOM input from afforestation and vegetation succession can improve both soil organic C and N dynamics. As shown in Table 6.2, there is a positive correlation between N_2 fixation and the C content of the soils from Chenqi catchment. This implies that higher SOC may result in more readily available energy supplies that are required by free-living diazotrophs for nitrogenase functioning. Meta-analysis studies have shown that enforcement of the Grain for Green programme has increased SOC in the top 20 cm layer by 48.1 % (Song *et al.*, 2014). This suggests that vegetation restoration reinforces SOM accumulation and hence the C content, thereby powering more BNF in the soil.

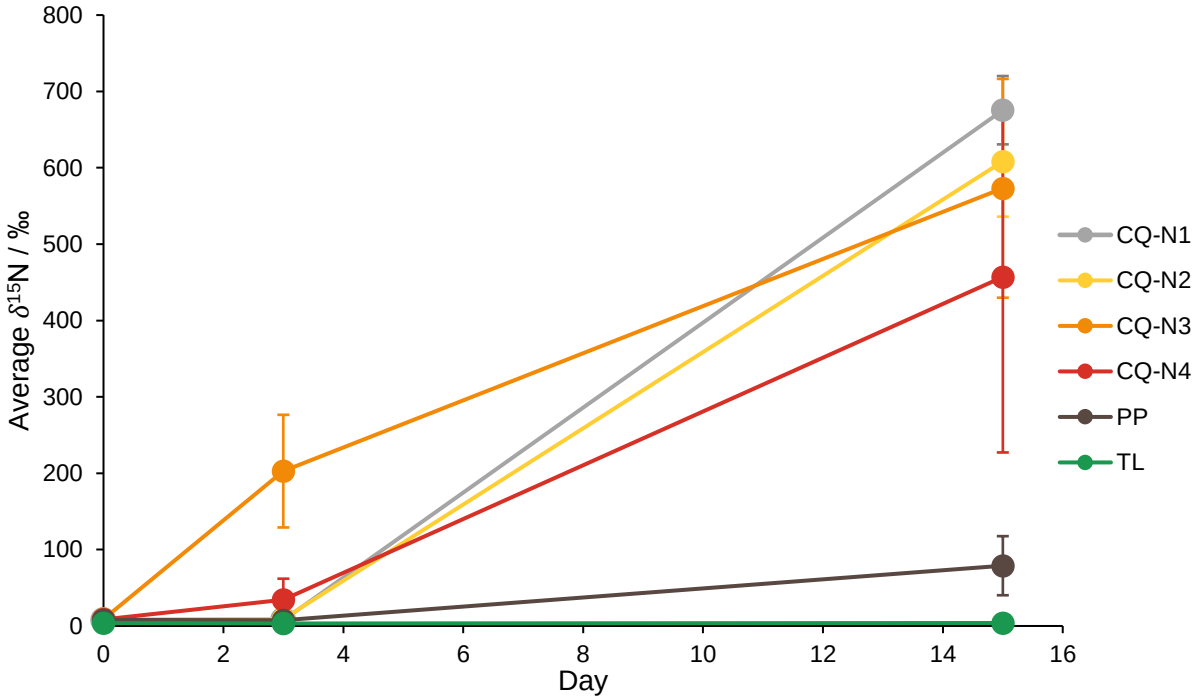


Figure 6.2. Mean bulk soil $\delta^{15}N$ values for soils from the Karst CZ, across various vegetation recovery phases. Energy was supplied on days 0 and 8 in the form of a RE mix. Error bars are ± SEM (n=3).

Table 6.2. Total N, total C (TC), soil organic C (SOC) and the C/N ratio for soils from the Chenqi catchment. Analyses were conducted on surface soils (0–20 cm). (Data from SPECTRA, 2019).

Land use	% TN	SOC / mg g^{-1}	C/N ratio
Cultivated field	0.18	13.77	10.29
Abandoned cropland (< 5 years)	0.23	17.60	10.33
Secondary forest	0.34	30.00	10.62

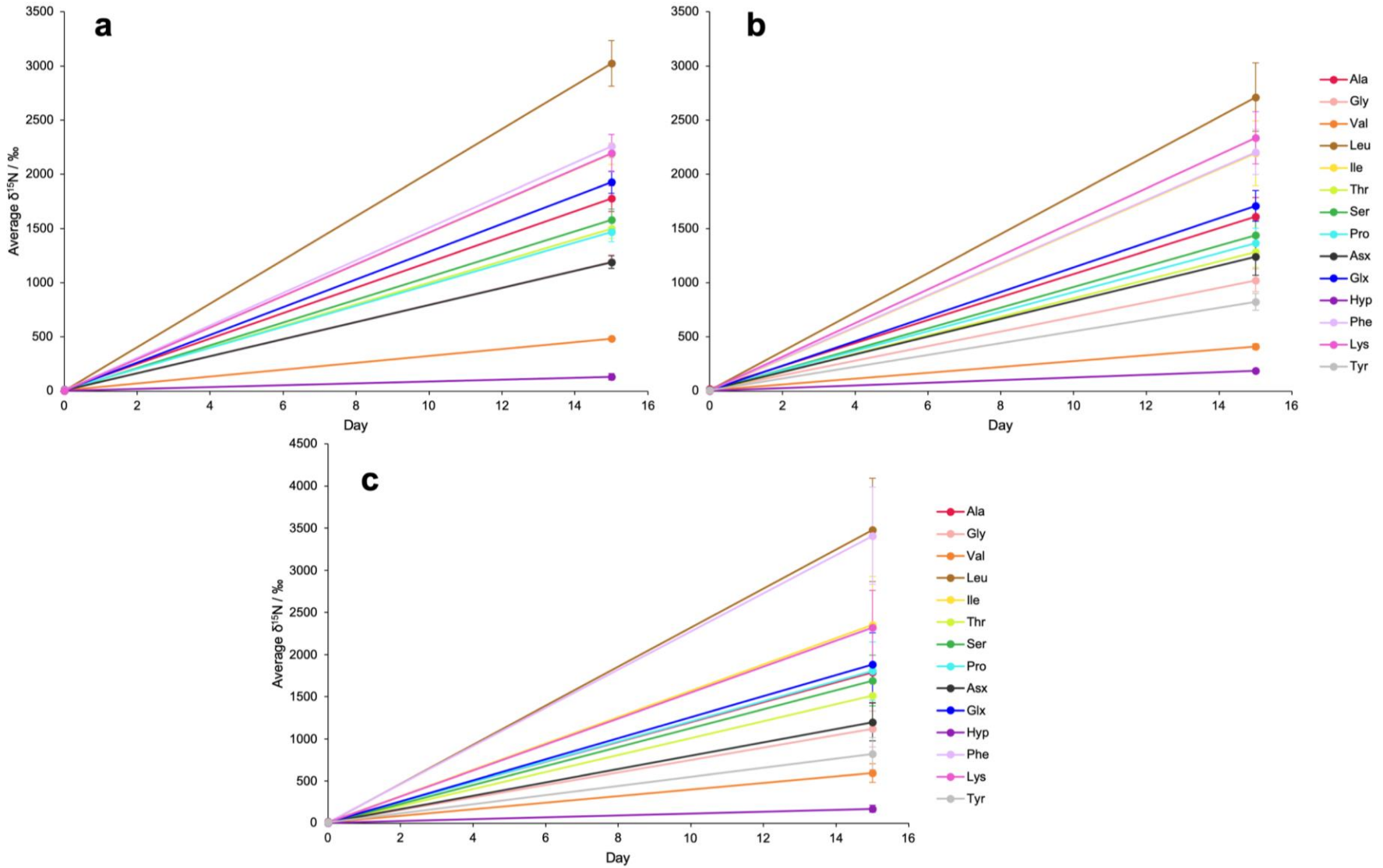


Figure 6.3. Average $\delta^{15}N$ values of individual AAs between control and day 15 samples for soils from the Karst CZ, under different stages of recovery: a) CQ-N1, b) CQ-N2, c) CQ-N3. Energy was supplied via a RE mix on days 0 and 8. Error bars are ± SEM (n=3).

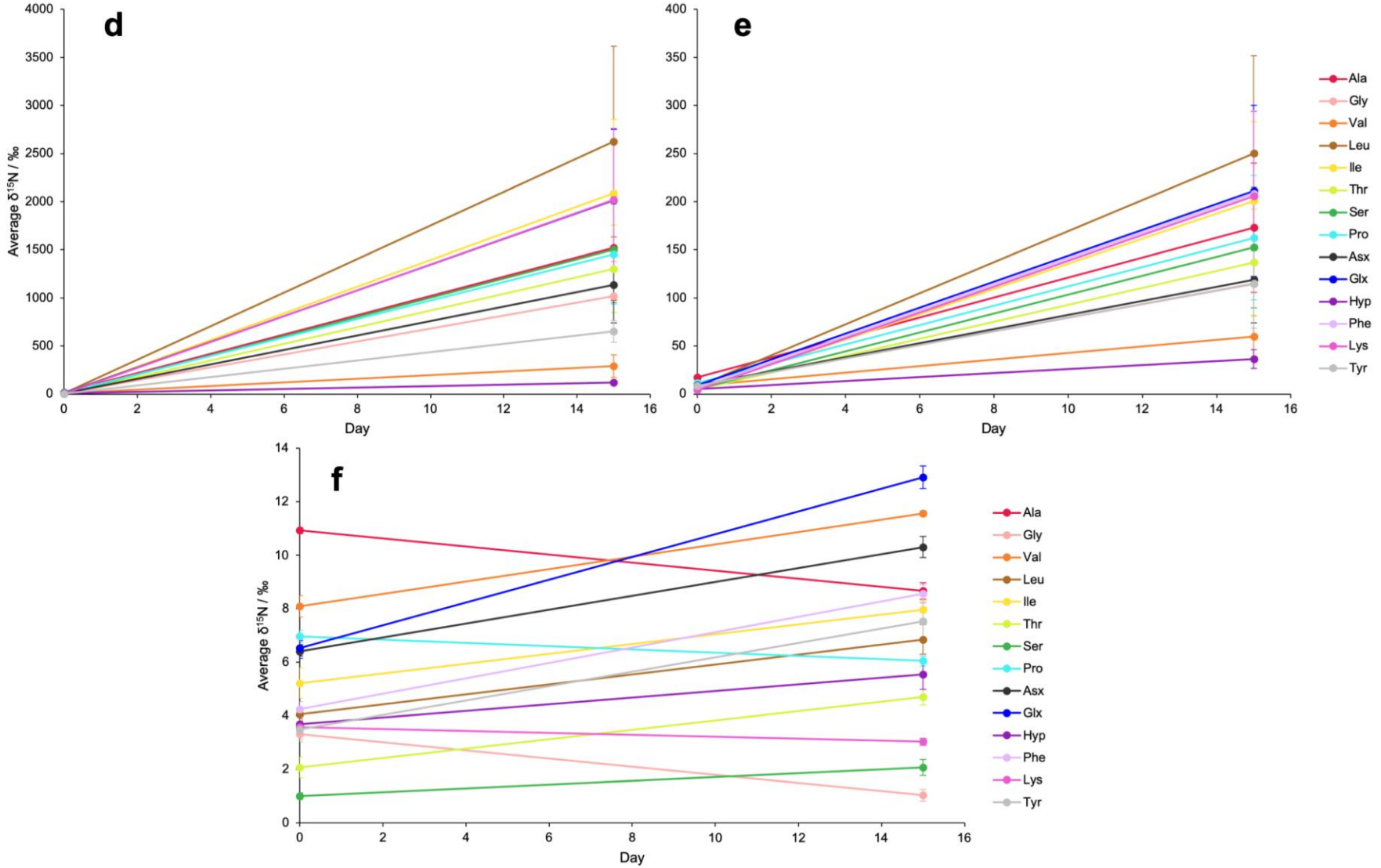


Figure 6.4. Average $\delta^{15}N$ values of individual AAs between control and day 15 samples for soils from the Karst CZ, under different stages of recovery: d) CQ-N4, e) PP, f) TL. Energy was supplied via a RE mix on days 0 and 8. Error bars are ± SEM (n=3).

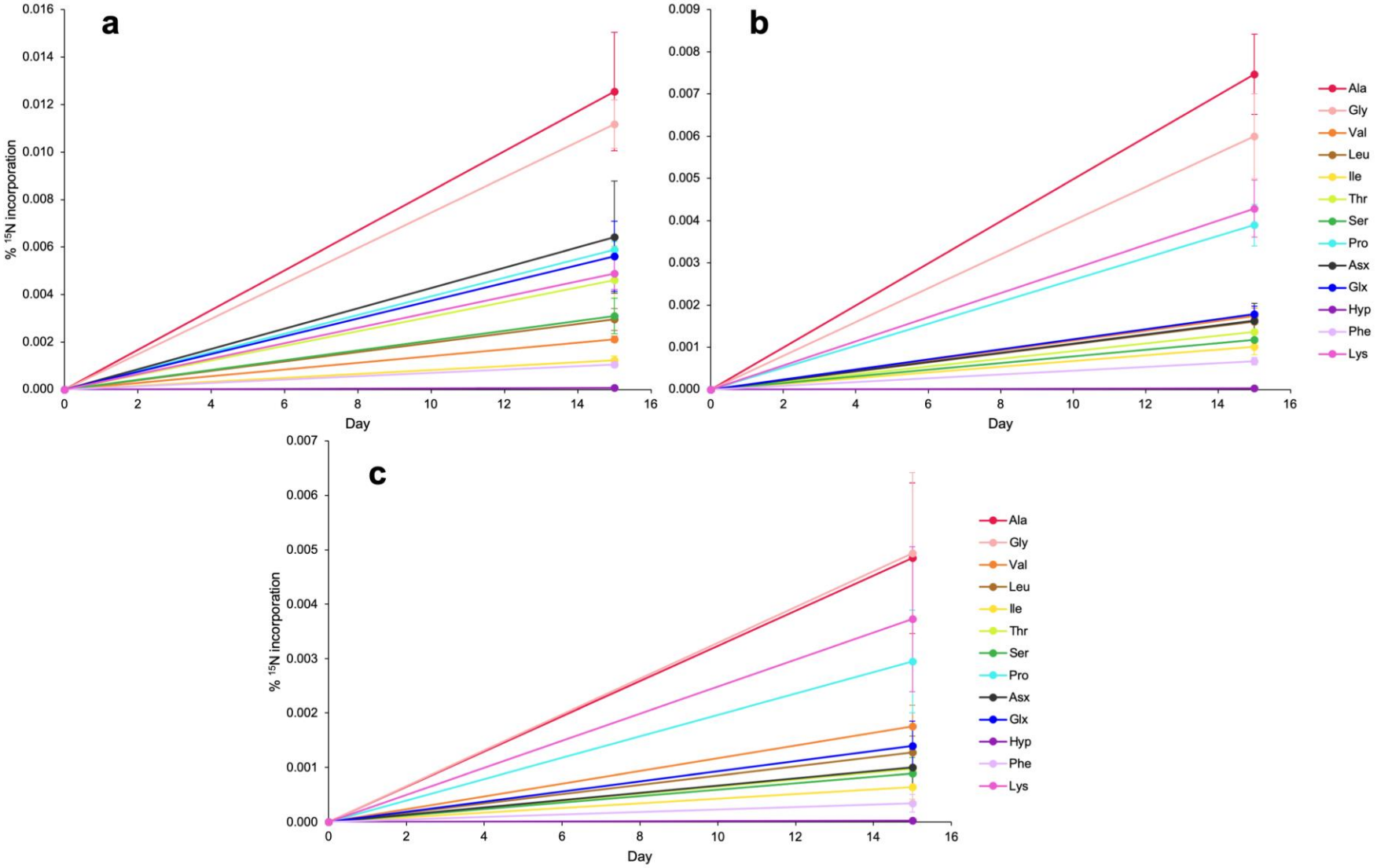


Figure 6.5. Average percentage applied ^{15}N incorporations (i.e. % N fixed) into individual hydrolysable soil AAs between control and day 15 samples for soils from the Karst CZ, under different stages of recovery: a) CQ-N1, b) CQ-N2, c) CQ-N3. Energy was supplied via a RE mix on days 0 and 8. Error bars are ± SEM (n=3).

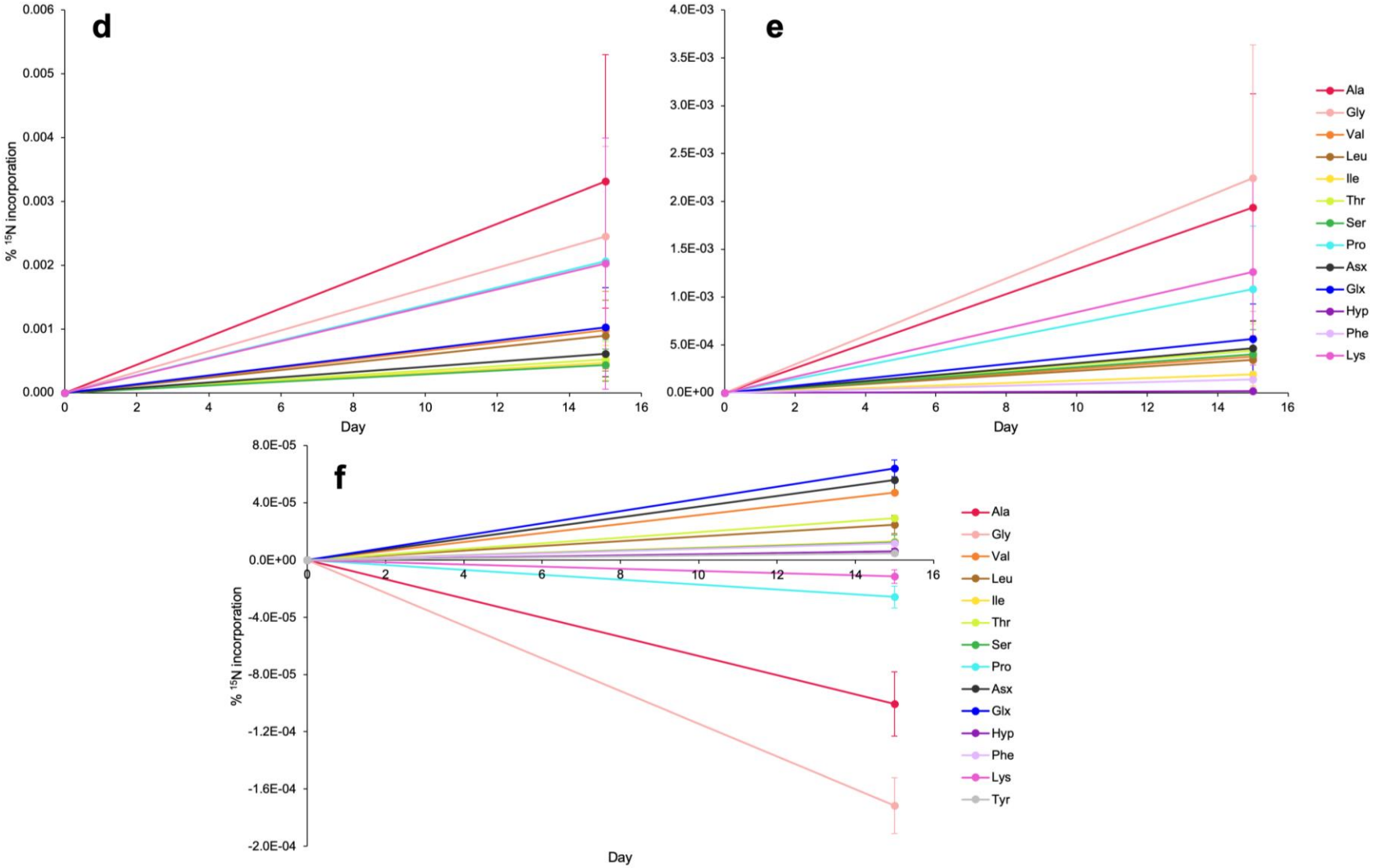


Figure 6.6. Average percentage applied ^{15}N incorporations (i.e. % N fixed) into individual hydrolysable soil AAs between control and day 15 samples for soils from the Karst CZ, under different stages of recovery: d) CQ-N4, e) PP, f) TL. Energy was supplied via a RE mix on days 0 and 8. Error bars are ± SEM (n=3).

Furthermore, the soils from CQ hold another interesting arrangement where N_2 fixation appears to decrease down the slope as well (i.e. higher upslope > upslope > valley; Fig. 6.7). The steep, sloping terrain of the karst landscape makes accelerated N loss a particular concern. Simulated rainfall experiments estimated a total N-loss load of 1.05–1.67 Tg N per year in the karst region of southwest China, with a majority of surface and subsurface runoff occurring during the monsoon season (Song *et al.*, 2017). Zhang *et al.* (2015) found both grasslands and shrublands to be constrained by N following agricultural abandonment in the karst area; Kang *et al.* (2015) revealed that cellular functions such as DNA synthesis in a group of karst plants were affected by N limitation. Taken together with our findings, it is suggested that BNF may also be promoted by general N deficiencies throughout the karst system, where a higher demand for N renders diazotrophs to perform fixation more actively.

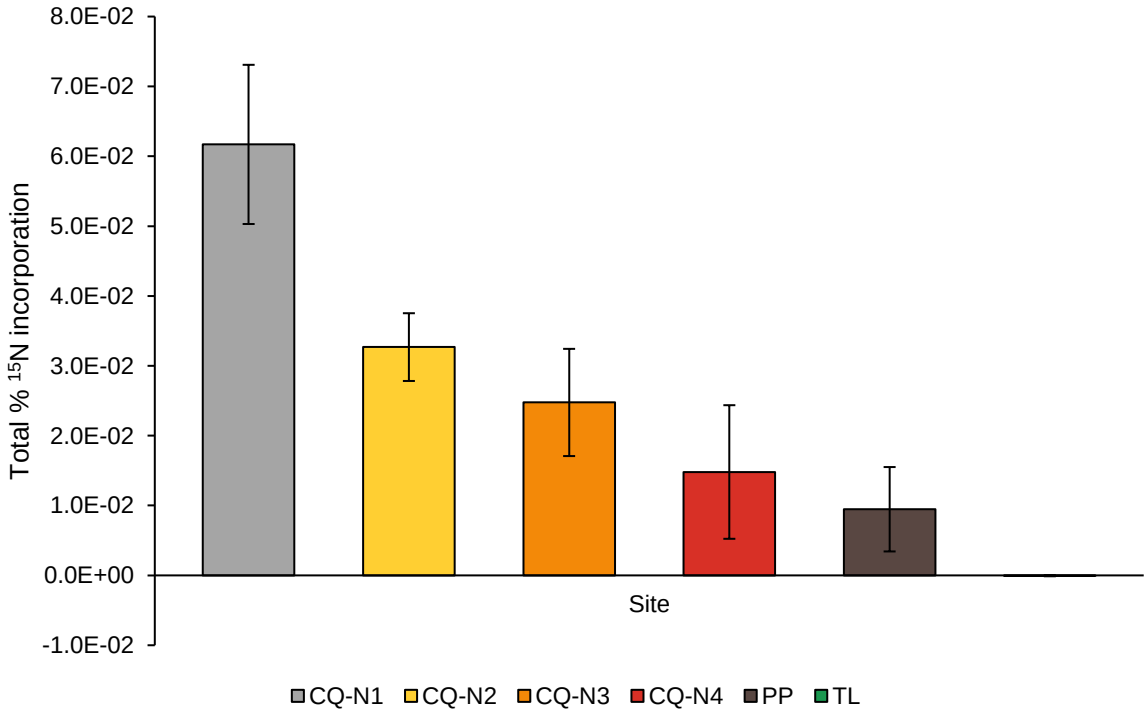


Figure 6.7. Total percentage applied ^{15}N incorporated into all hydrolysable soil AAs for soils from the Karst CZ, across various vegetation recovery phases. Error bars are ± SEM (n=3).

Surprisingly, soils from Tianlong Mountain did not show any diazotrophic activity – despite being a pristine primary forest site. As it happens, even compound-specific GC-C-IRMS analyses were unable to detect much applied ^{15}N incorporation into the TL soils (Fig. 6.6f); individual AA % incorporations cancelled each other out, resulting in a negative total value of $-(4 \times 10^{-5})$ % (Fig. 6.7). A possible explanation could be to do with the soil moisture content. Soil water

conditions play an influential role in driving microbial diversity across karst vegetation types by affecting the O_2 content and substrate availability in the system (Xue *et al.*, 2017). In our experiment, every soil sample received 3 mL of water at the start of the incubations. This amount was not adjusted to meet a specific level of moisture for each soil type and may have been insufficient for the TL soils. Maximum microbial activity is allegedly supported by 60 % water-filled soil pore space (Linn and Doran, 1984), while most effective fixation has been reported to occur under flooded conditions (Rao, 1978). On the other hand, atmospheric N deposition from abundant local urban and industrial emissions within the area can also lead to soil N accumulation (Wen *et al.*, 2016) and disregard the need for N_2 fixation.

6.4.2 Comparing experimental nitrogen fixation rates with existing *nifH* gene data

Analysis of N functional genes within the same sites by Li *et al.* (2018) found that the relative abundances of *nifH* were greatest in the primary forest soils, compared to other land recovery phases. In their study, the abundances of *nifH* genes increased in order of: recovering cultivated soils in Chenqi catchment < farmland < secondary forest < undisturbed soils at Tianlong (Fig. 6.8). The relationship between *nifH* gene abundances and vegetation recovery is rather unclear here, while also showing poor correlation with our experimental measurements of N_2 fixation (Fig. 6.7). This confirms that we cannot rely on genetic data and the presence of N functional genes alone to infer actual N-fixing activity.

It must be noted that the presence of *nifH* genes itself does not indicate active N_2 fixation. The process is tightly regulated on the microbial level and its expression may be subject to other environmental conditions (Bürgmann *et al.*, 2005). The porous limestone karst topography of the studied region is typically very poor in nutrients and a highly specialised microbial community has therefore evolved to accommodate this niche. In low-resource environments, previously cultivated soils exposed to repeated tillage, crop rotation and fertilisers may have consequently developed substantial microbial diversity (Xue *et al.*, 2017; Li *et al.*, 2018). These soils may actually hold a larger proportion of active N-fixing strains as higher N demand is required in intensive pasture areas to support crop growth. By comparison, although *nifH* gene abundances were greatest in the TL soils (Fig. 6.8), high-resource forest ecosystems are less inclined to changes in community composition and may accordingly have less active diazotrophs – thereby explaining lower rates of BNF observed in practice.

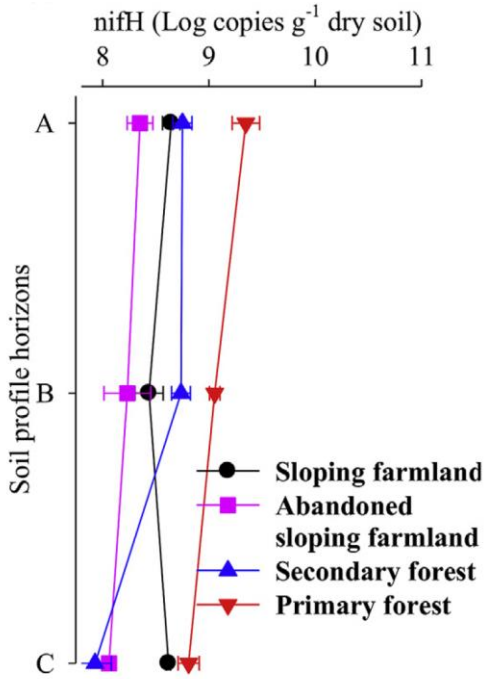


Figure 6.8. Absolute abundances of *nifH* genes (as log-*transformed* means) from each soil horizon for different vegetation recovery phases from the Karst CZ in southwest China. The sites are also aligned with our experimental sites, where sloping farming ≈ PP; abandoned sloping farmland ≈ CQ-N3; secondary forest ≈ CQ-N1; primary forest = TL. (Li *et al.*, 2018).

6.4.3 A new pathway of nitrogen assimilation by diazotrophs?

Results show that newly fixed N is largely incorporated into Ala – exposing an alternative pathway for N assimilation. In Figures 6.5 and 6.6, Ala displayed the highest % applied ^{15}N incorporation on day 15 for the majority of active N-fixing soils (i.e. soils from Chenqi catchment), and is followed closely by Gly. This indicates that, after fixation, there is a preferential distribution of fixed N into Ala over other AAs during N assimilation. Glu, assimilated via the GS/GOGAT pathway, is typically reported to serve as the central N metabolite for the biosynthesis of other AA compounds. This paradigm, however, shifted when Ala was revealed as the main N-containing compound excreted by symbiotic N-fixing soybean (Waters *et al.*, 1998) and pea bacteroids (Allaway *et al.*, 2000). The amount detected was also dependent on nitrogenase activity, where legume nodule bacteroids exposed to high levels of O_2 or to limited energy for N_2 fixation did not release Ala (Waters *et al.*, 1998). Together with our findings, these results suggest that Ala may have an important role in organic N assimilation and metabolism for both symbiotic and free-living diazotrophs.

Moreover, Ala has been shown as a principal accommodating AA when the soil microbial system is under certain stresses such as anaerobic conditions or flooding. Ala is formed via alanine dehydrogenase (ADH; Eq. 6.1) and has been suggested to accumulate under the anaerobic assimilation of NH_4^+. The reaction is revealed to be kinetically favoured as the pathway of C flow to Ala culminates in the production of ATP which meets the requirements of N assimilation, thereby allowing for energy to be maintained during anaerobicity (Vanlerberghe *et al.*, 1991). In the absence of O_2, pyruvate is also reportedly the only readily available C skeleton and is thus a key metabolic compound (Vanlerberghe and Turpin, 1990). Consequently, aside from serving as a transport species for fixed N, Ala may also perform other N metabolic functions not commonly mediated through Glu. Our developed method for the quantification of N_2 fixation, through the use of compound-specific AA ^{15}N-SIP, has allowed for additional biosynthetic pathways to be revealed. Nevertheless, the mechanisms of these processes require more extensive investigation as the regulation of nitrogenase activity also differs across distinct microbial groups in varied environments.

$$\text{L-alanine} + H_2O + NAD^+ \leftrightharpoons \text{pyruvate} + NH_3 + NADH + H^+$$

Equation 6.1. The chemical reaction catalysed by alanine dehydrogenase (ADH).

7 Overview

7.1 Have we fixed it?

The overarching aim of this work was the development of a novel compound-specific ^{15}N-SIP method to quantify BNF in soils. The potential of diazotrophs, especially the free-living sort, as a sustainable source of N input for crops and various landscapes has yet to be fully realised. This is largely due to the absence of sufficient reliable data available regarding their overall contribution to the biological input of fixed N. The full involvement of these N-fixers to soil fertility will be recognised with certainty only when accurate quantifications of N_2 fixation rates are in effect. Thus, valid and reliable measurements of N_2 fixation are crucial to testing hypotheses and thoroughly understanding controls regarding the process. Through this body of work, the ability of diazotrophs were evaluated through the development and application of our quantification method. The results of each chapter can be summarised into six points:

i) N_2 fixation is largely controlled by the nitrogenase enzyme and its physiological requirements. Through our methodology testing, N-fixing activity was substantially enhanced under anaerobic conditions and the exclusive supply of energy. This is reflected in the extremely O_2-sensitive property of nitrogenase, alongside highly demanding ATP requirements associated with N_2 fixation. Thus, a second injection of energy instantly stimulated a surge of fixation, which was further supported by the absence of O_2 within the soil incubation system.

ii) In terms of the energy source, it was found that a RE mix was more effective in fuelling N_2 fixation, than Glc alone. The diversity of compounds which make up RE increases the chances of meeting the C preferences of each individual diazotroph within an unknown soil microbial pool. Accordingly, N_2 fixation is often amplified in rhizosphere soil where labile C sources from RE are commonly found.

iii) Diazotroph activity is also controlled by the availability of N within a system. When fixed N is already available in the environment – for example, through inputs of both organic and inorganic N fertiliser, or atmospheric N deposition – diazotrophs can preferentially use it and may not necessarily perform N_2 fixation. This was largely

witnessed in the low amounts of fixed N in agricultural soils from both China and the UK, compared to those from more pristine sites. By contrast, the depletion or limitation of external stores of mineral N in a soil would preferentially encourage BNF.

iv) N-fixing activity and *nifH* gene abundances are generally positively correlated – however, it must be emphasised that the presence of nitrogenase functional genes itself does not denote N_2 fixation. Due to high energetic demands, the process is tightly regulated on the cellular level and its expression may depend on other environmental conditions. Genetic analyses alone therefore reflect an inexact representation of the functioning diazotroph community and their N-fixing activity. Rather, it could be more accurately implied that a higher abundance of *nifH* genes increases the probability of finding active strains of diazotrophs that will effectively engage in N_2 fixation.

v) Compound-specific ^{15}N-SIP using GC-C-IRMS is more sensitive and precise compared to bulk soil analyses. For all incubated samples, δ^{15}N values attained through compound-specific GC-C-IRMS nearly doubled those from the bulk approach. Most importantly, the lower detection limit of GC-C-IRMS allows soils with low N-fixing activity to be effectively quantified. This was most evident in Chapter 5 where the high sensitivity and selectivity of GC-C-IRMS was able to detect low levels of diazotrophic activity that was unattainable from bulk soil.

vi) Finally, compound-specific AA ^{15}N-SIP via GC-C-IRMS provides insights regarding the fate of the applied ^{15}N-tracer be gained. For the peat soils, ^{15}N incorporation into Glx dominated over other AAs (Chapter 4). Glu, catalysed by GS/GOGAT, is a main N-transporter and N-donor AA biosynthesised in the N assimilation pathway. An alternative pathway was revealed in the Chinese karst soils where major ^{15}N incorporation was seen in Ala (Chapter 6). Directed by the ADH enzyme, Ala ^{15}N-labelling was found to prevail in AAs under anaerobic conditions. Such new evidence emphasises the value of compound-specific GC-C-IRMS as a powerful tool for investigating biosynthetic pathways following the assimilation of fixed N into hydrolysable soil AAs.

To our knowledge, the work presented in this thesis was the first to utilise compound-specific ^{15}N-SIP via GC-C-IRMS to quantify BNF. It is also the first to study the biomolecular fate of fixed N going into the total hydrolysable AA or soil protein pool. Our developed method was able to successfully assay N_2 fixation in soils across an array of environments and land uses – we worked on a peatland, multiple grasslands and a recovering karst landscape, which were located in two distinct regions of different climatic conditions. High levels of ^{15}N enrichment also suggests the significant involvement of free-living diazotrophs in N_2 fixation. However, the abundance of regulators associated with the process also explain why N-fixers are not a dominant species in all systems where primary productivity is limited by N – and why N limitation is not necessarily alleviated by the presence of these microorganisms. The amount of N fixed by a soil microbial system is essentially non-random and can be estimated based upon habitat characteristics and management history, in relation to the key physiological controls directing nitrogenase activity. Optimal incubation conditions, leading to higher N_2 fixation, are met in the field presumably after rainfall events and during the warmer months when plant growth rates are high. Altogether, these factors and findings justify the validity of our quantification method and it can be said that we have, indeed, finally fixed it!

7.2 Practical method applications and future work

The developed method currently presents several practical applications. First, our method is required to know whether BNF has been optimised in a soil system. To better manage a field and improve the efficiency of BNF, less N fertiliser would have to be added. Thus, accurate and valid measurements of fixation are crucial to advise such strategies. N_2 fixation capacities (moles or mg N fixed g^{-1} soil) can now be expressed using our method; while this may not be a direct rate of fixation, it is similar to the way fertiliser application rates are expressed (e.g. kg N ha^{-1}). Second, our method can also be used as a way to assay the effectiveness of other methods of improving BNF. Genetic manipulation, for example, may lead to the development of new possibilities to increase BNF in soils – but none of it would be validated in absence of a quantification method to test the effectiveness of the engineered N functional gene.

Nevertheless, the developed method still lacks one final touch – it does not yet permit the measurement of BNF on an ecosystem scale. It is feasible to quantify rates of N accumulation in a sample over a relatively prolonged time or detect active nitrogenase enzymes through the use of our method – but to accurately quantify fixation in natural systems itself is more difficult. Most

studies, including this one, involve in vitro experiments on N-fixers. Rates of N_2 fixation in many biomes are in fact large-scale estimates representing significant extrapolations of a few point measurements (Cleveland *et al.*, 1999). Consequently, little is known about the actual performance of diazotrophs in natural ecosystems. The quantification of N_2 fixation should ideally occur *in situ*. In other words, a method without disturbances is required to verify the dynamics and patterns of fixation in the full extent of a field (Stewart, 1969; Knorr *et al.*, 2015). Methodological progress of this calibre could prompt a global re-evaluation of BNF and N cycling across various ecosystems.

There are possible ways to extend our work to improve its applicability in the real-world. Firstly, the sampling frequency could be increased. This entails sampling more locations within one site (e.g. sampling along a random 'W-shaped' transect within a field), as well as carrying out continuous sampling over an extended period of time. Larger sample distributions may reduce the inherent spatial and temporal variability of N_2 fixation, and biological systems in general, thereby enhancing our ability to assess inputs of fixed N on the ecosystem scale. A major problem also arises from the impracticality of using ^{15}N-labelled gas in the field as it would demand gastight enclosure of the entire system. Larger-scale incubations would also require substantial quantities of the isotopic gas, which is very costly. Results from $^{15}N_2$ incubation experiments on peat have been extrapolated to the field scale by utilising the density of peat at each depth (Knorr *et al.*, 2015). Another study conducted a 'pseudo-*in situ*' experiment where incubation vials containing *Sphagnum* were placed in the same location as where the samples were collected (i.e. incubations were out in a peatland itself), so that half the vial was kept above the water level (Larmola *et al.*, 2014). This may be an interesting direction to take as it offers practicality in terms of the $^{15}N_2$ gas demands, while also retaining some essence of an *in situ* experiment.

Another question that arises is – in what terms do our results provide the full N_2 fixation capacity of the incubated soil? For the peat, we could assume from the last two timepoints that the system has reached maximum capacity (BB1; Fig. 4.3 and 4.6); however, would another injection of the energy source stimulate further fixation? If so, when will the system run out of steam – i.e. at what point can it be said that the fixation capacity of a unit volume of soil has been measured? To our knowledge, there have not been any incubation experiments related to N_2 fixation, both here and in the literature, that have been carried out beyond 15 days. This would be something worth trying. In order to quantify the maximum N_2 fixation capacity of a weight of soil, we could carry out even longer incubations and continue to replenish the energy until N-fixing activity

comes to a complete stop. A problem that comes with prolonged incubations, however, is the need to also replenish the air within the vial. There are studies on soil methanotrophic bacteria which have progressed from static incubation chambers to a flow-through system, where a peristaltic pump was utilised to flow methane (CH_4) through the microcosms at a constant rate (Bull *et al.*, 2000; Maxfield *et al.*, 2006). But considering the expense of $^{15}N_2$, a recycling or recirculating gas system would have to be set up (Fig. 7.1). In order to deal with the anaerobic demand, a possible option would be to exclude O_2 from the system by using a mixture of $^{15}N_2$: Ar (or another inert gas). Furthermore, a syringe pump could also be used to add the RE mix at a low constant flow (e.g. µL min^{-1}) – in order to closely mimic the release of RE into the soil rhizosphere. On the whole, numerous opportunities exist for the extension of the developed method to obtain a more accurate 'real-world' rate of N_2 fixation.

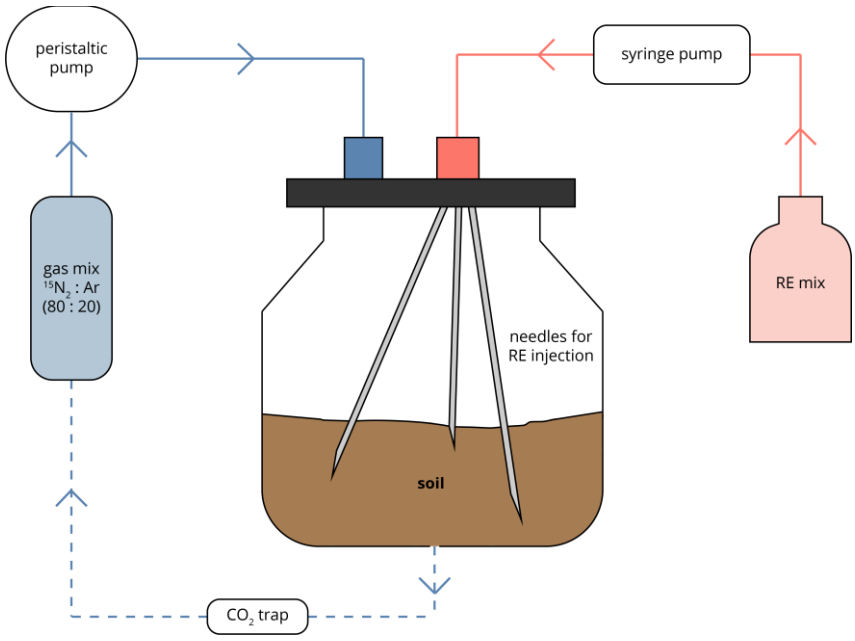


Figure 7.1. A basic set-up for the proposed flow-through incubation system. Only the top of the vial is sealed to allow gas flow; the blue lines represent the direction of flow as the $^{15}N_2$ gas mix is recirculated. The suggested pathway for the addition of the RE mix is depicted by the red lines. Inside the incubation vial, the injection needles are positioned so that the RE is dispersed into the soil layers as evenly as possible.

7.3 Concluding statement

Overall, the successful development of a novel compound-specific ^{15}N-SIP via GC-C-IRMS method to quantify BNF contributed to an improved interpretation of the process and its controlling factors, in a highly sensitive manner. Even so, a more complete application on the wider ecosystem scale needs to be further established. BNF, however, cannot be the Holy Grail, or the all-encompassing solution to our challenge for sustainable food security. An integrated multidisciplinary approach is necessary to secure soils and emphasise their role in providing valuable ecosystem services. From another perspective, despite significant increases in agricultural production, one in eight people living in developing countries still remain malnourished (FAO, 2015). This calls into question whether the problem lies in limited natural resources or in the unrelenting needs of the people.

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